AD_____

Award Number:
W81XWH-06-1-0406

TITLE:
New Advances in Molecular Therapy for Muscle Repair after Diseases and Injuries

PRINCIPAL INVESTIGATOR:
Johnny Huard, Ph.D.

CONTRACTING ORGANIZATION:
Children's Hospital of Pittsburgh of
UPMC Health System

REPORT DATE:
April 2010

TYPE OF REPORT:
Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-Apr-2010	Annual	3 MAR 2009 - 2 MAR 2010

4. TITLE AND SUBTITLE
New Advances in Molecular Therapy for Muscle Repair after Diseases and Injuries

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0406

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Johnny Huard, Ph.D.

Email: jhuard@pitt.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Children's Hospital of Pittsburgh of UPMC Health System
Pittsburgh, PA 15224

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

See next page.

15. SUBJECT TERMS

None provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	217	19b. TELEPHONE NUMBER *(include area code)*

14. Abstract

Project #1

Background: Muscle injuries, especially pulls and strains, are among the most common and most frequently disabling injuries sustained by athletes and soldiers. Although injured muscles heal naturally, the regeneration is very slow and often yields incomplete functional recovery. In injured muscle, regeneration begins shortly after injury, but the healing process is rather inefficient and is hindered by fibrosis—that is, scar tissue formation. More importantly, the scar tissue that often replaces damaged myofibers may contribute to the tendency of strains to recur. We have observed that TGF-β1 plays a central role in skeletal muscle fibrosis and, more importantly, that the use of antifibrosis agents that inactivate this molecule, such as suramin (a Food and Drug Association [FDA]-approved drug that prevents fibrosis due to skin disorders), can reduce muscle fibrosis and consequently improve muscle healing, resulting in nearly complete recovery after laceration or strain injuries.

Objective/Hypothesis/Specific Aims/Study Design: We plan to develop biological approaches based on suramin to efficiently prevent the scarring process by blocking the action of TGF-β1; we will determine the appropriate time at which to administer suramin and the optimal suramin dosage after muscle contusion, a common military injury (**Technical Objective #1**). Because we have observed that suramin can both enhance muscle regeneration and neutralize the fibrotic effect of TGF-β1, we also propose experiments designed to further evaluate the beneficial effects of suramin on muscle regeneration (**Technical Objective #2A**). Finally, we will determine if this effect is mediated through suramin's interaction with muscle growth regulators, particularly its possible down-regulation of myostatin or up-regulation of follistatin (**Technical Objective #2B**).

Supplemental proposal 1 Objectives:

Supplemental Objective 1: To determine the pharmacokinetics of suramin delivery when administered via intramuscular injection in mice for the treatment of skeletal muscle injuries.

Supplemental Objective 2: To evaluate the beneficial effect of decorin, another antifibrosis agent, on muscle regeneration and healing and its mechanism of action.

Supplemental proposal 2 Objectives:

Supplemental Objective 1: To evaluate whether decorin's beneficial effect on muscle healing is mediated through its influence on muscle inflammation. **Hypothesis:** Decorin improves muscle healing by influencing muscle inflammation.

Supplemental Objective 2: To investigate whether decorin's beneficial effect on muscle healing is mediated through MSTN or FLST, two important regulators of muscle growth. **Hypothesis:** Decorin promotes muscle healing by down-regulating MSTN or up-regulating FLST.

Supplemental Objective 3: To evaluate whether angiotensin II receptor blockade after injury represents a potential non-invasive approach to improve muscle regeneration and repair after military-related muscle injury
Hypothesis: Angiotensin II receptor blockade will improve muscle healing after injury.

Relevance: These studies should further our understanding of the muscle healing process, facilitate the identification of new techniques to promote efficient muscle healing, and contribute to the development of innovative therapies for various types of muscle injuries and diseases, such as muscular dystrophies.

Project #2

Background: Muscle injuries are very common musculoskeletal problems in traumatology that arise frequently in military training and combat. Injured skeletal muscle undergoes a natural process of regeneration; however, fibrosis—the formation of fibrous scar tissue—hinders this process and precludes the complete recovery of muscle function. Prevention of fibrosis could improve injured skeletal muscle healing; however, it often is not possible to treat muscle injuries before fibrosis occurs.

Objective/Hypothesis: We hypothesize that matrix metalloproteinase type 1 (MMP1), a collagen-digesting enzyme, can improve the microenvironment for muscle regeneration and accelerate healing by digesting existing scar tissue within recovering muscle. Further we hypothesize that MMP-2 can enhance muscle cells migration and regeneration, reorganize muscle structure and accelerate muscle healing after injury.

Specific Aims/Study Design: First, we will culture muscle-derived fibroblasts and myogenic cells in vitro in the presence of MMP1 and assess its effects on fibroblast proliferation and collagen deposition (**Technical Objective #1A**) and myogenic cell migration and differentiation (**Technical Objective #1B**). We then will inject MMP1 directly into normal (noninjured) skeletal muscle to determine the safest and most effective dose of MMP1 in vivo (**Technical Objective #2A**). Next, we will evaluate the ability of MMP1 to digest fibrous scar tissue present within injured skeletal muscle and, by so doing, to enhance the regeneration and functional recovery of injured skeletal muscle (**Technical Objective #2B**). Finally, we will attempt to extend the effective half-life of MMP1 in fibrous scar tissue by genetically engineering myoblasts and muscle-derived stem cells (MDSCs) to express MMP1 (**Technical Objective #3**).

Supplemental Proposal 1 Objectives:

We will evaluate the effects of MMP-2 on myogenic cell migration and differentiation *in vitro* (**Technical Objective #1**). We then will use MMP-2 enhance muscle cells migration and regeneration to improve the functional recovery of injured skeletal muscle *in vivo* (**Technical Objective #2**). Further, we will attempt to assess the relationship between MMP-1 and MMP-2 during muscle healing (**Technical Objective #3**).

Relevance: The study results generated by the proposed project should shed further light on the effects of scar tissue on muscle healing after injury and could facilitate the development of methods by which to eliminate scar tissue and enhance the regeneration of muscle damaged by military- or sports-related injuries or diseases.

Project #3

Our preliminary studies point to a close developmental relationship between vascular endothelial cells and myogenic cells in the adult human muscle. We have, indeed, characterized two novel populations of muscle-derived, non-satellite cells that exhibit dramatic myogenic potential in culture and *in vivo:* genuine vascular endothelial cells and cells co-expressing markers of both myogenic and endothelial cell lineages, which we have named myo-endothelial progenitors. Both cell populations can be purified by flow cytometry as endothelial (CD56- CD 34+ CD144+) and myo-endothelial (CD56+ CD34+ CD144+), and cultured for several weeks without losing their myogenic potential. In our preliminary experiments, the myogenic potential in vivo of myo-endothelial progenitors is dramatically higher than that of endothelial cells, which are themselves much more efficient than regular myogenic cells. We propose experiments to further assess the respective myogenic potentials of human muscle-derived endothelial and myo-endothelial cells, whch will be compared qualitatively and quantitatively following injection into the injured SCID mouse muscle. Besides myogenesis, the development of other cell types, notably endothelial cells and pericytes, upon intramuscular injection will be examined. We hypothesize that upon myo-endothelial cell transplantation, high rates of donor cell survival and/or proliferation will promote skeletal muscle regeneration by generating more donor cells that can subsequently participate in the regeneration process. We propose a set of experiments to determine the rate at which these different populations of human muscle-derived cells survive and proliferate at different time points after injection, and to evaluate if such differences might affect the regeneration capacity of the cells after transplantation into the skeletal muscle. We will also perform experiments to investigate if these various human muscle-derived cell populations implanted in mice *via* intramuscular injection undergo self-renewal. We will also use a previously described protocol to determine if the donor-derived muscle precursor cells isolated from the skeletal muscle of primary recipient mice can be re-transplanted into the skeletal muscle of secondary recipients and improve skeletal muscle regeneration. To this aim, we will retrovirally modify human-derived myogenic, endothelial and myo-endothelial cells to express GFP. This will enable us to rapidly count the GFP-expressing cells isolated from primary recipients and sort them with high purity for re-transplantation into

secondary recipients. Finally, we will also check if cell fusion between donor cells or between donor and host cells plays a significant role in muscle regeneration by these different subsets of human cells.

Supplemental Proposal 1 Objectives:

1- Characterize the potential of pericytes to give rise to a full array of differentiated cells following transplantation into the injured skeletal muscle. We will determine, in a xenochimeric model, whether pericytes can, besides myofibers, regenerate other cell compartments that are fundamental to wound healing and tissue regeneration: blood vessels, connective tissues, nerves.
2- Determine the potential of pericytes to give rise to more primitive stem cells (auto-renewal), and/or to committed muscle progenitor cells (satellite cells) on transplantation into the injured skeletal muscle. Long-term and iterative transplantation experiments will be performed in order to understand whether pericytes can not only regenerate skeletal muscle, but do it permanently. This is a key point to be taken in consideration in the perspective of transplanting these cells into patients.
3- Master the long-term culture of pericytes while maintaining their developmental potential intact. The biology of pericytes *in vitro* will be scrutinized in order to validate culture protocols allowing to obtain large lots of transplantable cells.

Project #4

Background: As progress toward understanding the basic biology of stem cells continues to grow, it is of vital importance that researchers maintain a focus on therapeutic applications of this technology by investigating preclinical models. Members of our laboratory have identified a mouse muscle-derived stem cell (MDSC) population that exhibits a highly enhanced ability to regenerate skeletal muscle in a muscular dystrophy model. Transplantation of this cell population results in significantly more efficient regeneration of skeletal muscle fibers and a significantly larger area of regeneration than does myoblast transplantation, a therapy that has already been tested in human clinical trials in both the United States and Canada. The isolation and transplantation of the human equivalent of these mouse MDSCs likely would improve the outcome of cell therapy for muscular disease and injury, including injuries frequently sustained by military personnel.

Objective/Hypothesis: The objectives of this project are 1) to identify the human stem cell populations that promote the most efficient skeletal muscle regeneration in a preclinical mouse model of muscle regeneration and Duchenne muscular dystrophy (DMD) and 2) to identify the optimal conditions under which to expand cell populations and obtain therapeutically relevant doses.

Study Design: We will screen human muscle-derived cells for a molecular and behavioral profile that correlates with efficient skeletal muscle regeneration in a preclinical model of DMD (Technical Objective #1). We also will identify the cell culturing conditions that best facilitate expansion of the potent populations (to therapeutically useful quantities) while maintaining the cells' phenotype and regeneration efficiency (Technical Objective #2).

Supplemental Proposal 1 Objectives: While ongoing projects are focused on characterizing how to identify and maintain the stem cell phenotype of human MDSC, we are also initiating studies to identify optimal conditions for the cryostorage of these cells. We will build on the Live cell imaging (LCI) technology as a tool identify the optimal conditions for the processing of the cells as a biologic product for therapeutic applications. LCI provides live viewing of the behavior of a stem cell population in culture and allows us to obtain numerous real-time measurements at the single cell level. The result is a detailed behavioral phenotype of the stem cell population which can be linked to in vivo outcome measures. We will use this tool to follow the changes of cell phenotype in response to temperature changes and fluctuations.

Relevance: This project represents a critical step in cell therapy: Screening potential cell candidates and studying expansion kinetics and limits to generate stem cells for use in cell and gene therapy. This project is unique in that it takes the next step in moving muscle stem cells toward clinical application by investigating how to develop a safe and standardized approach by which to expand stem cell populations. The stem cell

screening and expansion techniques that we plan to develop will facilitate the use of cell and gene therapy for myriad musculoskeletal injuries and diseases.

Project # 5

Congenital muscular dystrophy (CMD) is a group of severe forms of muscular dystrophy leading to early death in human patients. The majority of cases are caused by genetic mutations in the major laminin that contains the α2 chain (formerly named merosin) in the muscle basement membrane. The early morbidity/fatality and the lack of effective treatment require urgent search for novel therapeutics. Previously, we utilized mini-agrin, which has been proven to have a therapeutic effect in transgenic MCMD mice, to treat MCMD mice by AAV vector. Our preliminary studies showed that over-expression of mini-agrin protein by AAV vector greatly improved general health and muscle morphology in MCMD mice. However, the treated disease mice still developed gradual paralysis and displayed shorter life span than wild type mice. To further improve the current gene therapy paradigm, with the advanced AAV technology and muscle biology knowledge, we will vigorously test our hypothesis: whether muscle pathogenesis can be improved by inhibition apoptosis or promoting muscle growth. The specific aims are the following:

Aim1: To investigate whether muscle pathogenesis can be improved by delivery of BCL2, an anti-apoptotic gene, by AAV vector in MCMD mice. Mice that lack laminin α2 show severe muscle loss, poor regeneration, and a greatly shortened lifespan. A role for apoptosis in pathology of laminin α2-deficiency has been suggested by histological and in vitro studies, as well as transgenic studies. In this study, we will explore the potential therapeutic effect by delivering AAV-BCL2 vector into MCMD mice.

Aim2: To examine whether therapeutic effect can be obtained by delivery of insulin like growth factor 1 (IGF-1) gene, which can promote muscle growth, by AAV vector in MCMD mice. The MCMD mice show muscle atrophy and enhanced fibrosis as seen in human patients. Genes that promote muscle growth and inhibit fibrosis is theoretically beneficial for congenital muscular dystrophic muscle. Myostatin blockade, one of the strategies to promote muscle growth, has been shown to have a severe side effect of increasing postnatal lethality in MCMD transgenic studies. The reason for the side effect is due to significantly less brown and white fat in the absence of myostatin. In our preliminary studies, we observed that myostatin blockade significantly increased muscle weight, as well as decreased fat tissue in normal mice. However, over-expression of IGF-1 in normal mice only increased muscle weight without loosing fat. Considering less fat will result a severe side effect, we will deliver IGF1 gene to MCMD mice by AAV vector to study whether a therapeutic effect can be achieved in this proposal.

Upon completion, this project will establish complementary therapeutic strategies to combat the severe congenital muscular dystrophy in animal model, setting the base for the development of a clinically efficacious gene therapy strategy.

Project # 6

Background: Elegant studies show that certain cytokines trigger the activation of nuclear factor kappaB (NF-kB) mediated by phosphorylation and degradation of the NF-kB inhibitory protein, IkappaBalpha. Downstream effects of pathological NF-kB activation in skeletal muscle include the inhibition of new muscle formation and the degeneration of existing muscle. *In vitro* studies support the potential that the IkappaBalpha superrepressor (IKBSR), an IkappaBalpha genetically engineered to prevent its phosphorylation, can prevent the activation of NF-kB in skeletal muscle and could ameliorate or prevent muscle wasting. Our preliminary studies demonstrate the novel determination of inhibition of activation of NF-kB by cFLIP.

Objective/Hypothesis: The proposal will explore the mechanism of muscle wasting in an *in vitro* model and develop novel gene transfer vehicles testing the hypothesis that gene transfer strategies can promote muscle regeneration toward a goal of improving muscle bulk and strength in the setting of injuries or diseases that cause muscle atrophy.

Specific Aims:

Aim 1: To characterize an *in vitro* model of cancer-induced muscle wasting in primary muscle cells and in stable muscle cell lines expressing IKBSR or cFLIP.

Aim 2: To clone, rescue, and purify AAV serotype 1 vectors carrying IKBSR or cFLIP and characterize expression and function *in vitro* in anticipation of future use in an *in vivo* model of muscle wasting.

Study Design: We will characterize an *in vitro* model of muscle wasting studying NF-kB activation, the ubiquitin-proteasome system and caspase activation. We will test whether genetic modifications to muscle cells will confer a benefit by blocking pathways of muscle degeneration. We will develop gene transfer vectors designed to promote muscle regeneration.

Supplemental Proposal 1 Objectives:
Supplemental Objective 1: To test gene transfer approaches in an *in vivo* model of cachexia
A. Test AAV carrying IKBSR for treatment of cancer cachexia
B. Test AAV carrying cFLIP for treatment of cancer cachexia
Supplemental Objective 2: To test peptide transduction approaches of inhibiting NF-κB activation as treatment for muscle cachexia
A. Test peptide transduction in an *in vitro* assay of muscle cachexia
B. Test peptide transduction in an *in vivo* assay of muscle cachexia

Relevance: The ability to promote muscle regeneration in the setting of focal or generalized muscle loss could confer significant clinical benefit in the setting of focal neuropathic or other processes that cause muscle atrophy or chronic illnesses that cause cachexia.

Vector Core:
Background:
The newly established molecular biology laboratory (MBL) will be a Vector Core for the project "Molecular Therapy of Muscle Repair". The goal of this core is to construct vectors such as adeno-associated-, adenoviral- and retoviral vectors for this project. Besides the tissue specific promoter and optimal serotype of vectors in the project #5 and project #6, we will develop a newly modified AAV vector, called the Tet/on and Tet/off AAV vector. This new vector is very useful to ameliorate the acute inflammatory during the early time of muscle injuries. The gene in Tet/on vector will be induced to express the therapeutic gene in the presence of doxycycline, an antibiotic. To avoid the toxicity caused by gene over expression, the vector will be turned off at the time when the muscle is healed.
Objectives:
Objective 1: To continue the production of AAV vectors for the following genes
A: Decorin for project #1
C: Mini-dystrophin gene
D: MPRO (myostatin propeptide) for project # 5
Objective 2: To construct the following genes into AAV vector
A: MMP1 for project #2
B: IKBSR for project #6
C: cFLIP for project #6
Objective 3: To design the new AAV vector containing muscle tissue specific promoter.
Objective 4: To develop the adenoviral and retroviral vectors.

BioReactor Core
The Bioreactor Core serves as a resource for project investigators in need of dynamic cell population analyses. This core uses the Automated Cell CytoWorks™ (ACCW) robotic system to characterize and compare various populations of adult-derived stem cells. The system, in theory, can give the investigator a read out of 20 to 30 different parameters in the form of a "Phenoprint©". We have also planned to set up internet technology to share the information generated in the core facility and thus facilitate further study and development of adult-derived stem cell–based therapies by members of the broader scientific community.

15. SUBJECT TERMS					
Project #1: Muscle Injuries, suramin, Fibrosis, TGF-beta1, myostatin, follistatin, decorin, Losartan Project #2: Muscular dystrophy, AKT1/Foxo/atrogin1 pathway, glucocorticoids Project #3: Muscle, stem cell, vascular endothelium, tissue regeneration, cell therapy Project #4: muscle regeneration, human myogenic stem cells, expansion, cell aging, population doubling, fibrosis Project #5: Adeno-associated viral vector (AAV),BCL-2, IGF-1, Laminin, myostatin, congenital muscular dystrophy Project #6: cachexia, muscle, cancer, trauma, adeno-associated virus, gene transfer					
16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** Johnny Huard
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**		59 plus 18 Appendices	**19b. TELEPHONE NUMBER** *(include area code)* 412-648-2798

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Project # 1 Progress Report
The use of suramin to improve muscle healing after military-related muscle injuries

(Johnny Huard)

Introduction

Muscle injuries, especially pulls and strains, are among the most common and most frequently disabling injuries sustained by athletes and soldiers. Although injured muscles heal naturally, the regeneration is very slow and often yields incomplete functional recovery. In injured muscle, regeneration begins shortly after injury, but the healing process is rather inefficient and is hindered by fibrosis—that is, scar tissue formation. More importantly, the scar tissue that often replaces damaged myofibers may contribute to the tendency of muscle strains to recur. We have observed that TGF− β1 plays a central role in skeletal muscle fibrosis and, more importantly, that the use of antifibrosis agents that inactivate this molecule, such as suramin (a Food and Drug Association [FDA]-approved drug that prevents fibrosis due to skin disorders), can reduce muscle fibrosis and consequently improve muscle healing, resulting in nearly complete recovery after laceration or strain injuries.

The studies proposed in Technical Objectives 1, 2A and 2B will enable us to determine whether suramin, a drug already approved by the FDA, can improve muscle healing by preventing scar tissue formation after a common military injury (muscle contusion); we also will examine the mechanism(s) by which suramin promotes muscle regeneration. The results of these studies could aid in the development of innovative therapies to promote healing in muscles damaged by different types of injury or disease (e.g., muscular dystrophies), therapeutic approaches that could easily be translated from animal experiments to human clinical applications.

In Supplemental Proposal 1, New Technical Objective 1 we will perform pharmacokinetic studies in mice to evaluate the concentration/ time data of suramin when administered intramuscularly. Our laboratory has shown that intramuscular (IM) injection of suramin after laceration and strain injuries decreases skeletal muscle fibrosis in mice and improves muscle healing, resulting in a near complete recovery of muscle function. In New technical objective 2 we will evaluate the beneficial effect of that decorin, another antifibrotic agent, has on muscle regeneration and healing and its mechanism of action. In preliminary studies we have observed that decorin enhances myogenic differentiation of myoblasts (C2C12 cells) in vitro and that C2C12 cells genetically engineered to express decorin display an enhanced ability to undergo myogenic differentiation in vitro and in vivo. Decorin has also been shown to neutralize the fibrotic effect of TGF-β1 and independently enhance muscle regeneration.

We have observed that direct gene transfer of decorin by injection into uninjured skeletal muscle can decrease the fibrosis and enhance the muscle regeneration observed after subsequent injury of that muscle. In Supplemental Proposal 2, New Technical Objective 1 we will evaluate whether decorin's beneficial effect on muscle healing is mediated through its influence on muscle inflammation. It has been observed that decorin influences the differentiation, survival, and infiltration of macrophages. We, therefore, propose experiments designed to reveal the role of decorin on the inflammatory phase of muscle healing. In Supplemental Proposal 2, New Technical Objective 2 we will also investigate whether decorin's beneficial effect on muscle healing is mediated through MSTN and/or FLST, two important regulators of muscle growth that have been shown to be integral to skeletal muscle regeneration processes. In Supplemental Proposal 2, New Technical Objective 3 we will evaluate yet another antifibrotic agent Losartan. Losartan is an angiotensin II receptor blocker which is cliniocally used for the treatment of hypertensive disorders which was shown to have the unexpected side effect of reducing muscle wasting in these patients. We have preliminary results that demonstrate a beneficial effect form Losartan on skeletal muscle after injury and in this 3rd objective will further study angiotensin II blockade as a potential non-invasive approach to improve muscle regeneration and repair after military-related muscle injury

Body

1) Original Proposal Objectives

Technical Objective 1 : Using an animal model of muscle contusion, a common military injury, we will determine the appropriate dose and time of administration of suramin.

Hypothesis: This is a non-hypothesis driven objective that will rely on empirical data results

Technical Objective 2A: Characterization of the mechanisms by which suramin enhances muscle regeneration will be explored in order to reveal novel ways to promote muscle growth and regeneration.

Hypotheis: Suramin will be shown to effect the key fibrosis and muscle regeneration triggers in skeletal muscle.

Technical Objective 2B: We will investigate suramin's interaction with myostatin and follistatin, 2 important regulators of muscle growth.

Hypothesis: Suramin will down-regulate the expression and up-regulate the expression of follistatin in injured skeletal muscle, thereby improving muscle regeneration and repair.

2) Supplemental Proposal 1 Objectives:

New Technical Objective 1*:* To determine the pharmacokinetics of suramin delivery when administered via intramuscular injection in mice for the treatment of skeletal muscle injuries.

Hypothesis: This is a non-hypothesis driven objective that will rely on empirical data results

New technical objective 2*:* To evaluate the beneficial effect of decorin, another antifibrosis agent, on muscle regeneration and healing and its mechanism of action.

Hypothesis: Decorin will increase muscle regeneration and repair through the enhancement of muscle regeneration and reduction fibrosis.

3) Supplemental Proposal 2 Objectives:

New Technical Objective 1: To evaluate whether decorin's beneficial effect on muscle healing is mediated through its influence on muscle inflammation.

Hypothesis: Decorin improves muscle healing by influencing muscle inflammation.

New Technical Objective 2: To investigate whether decorin's beneficial effect on muscle healing is mediated through MSTN or FLST, two important regulators of muscle growth.

Hypothesis: Decorin promotes muscle healing by down-regulating MSTN or up-regulating FLST.

New Technical Objective 3: To evaluate whether angiotensin II receptor blockade after injury represents a potential non-invasive approach to improve muscle regeneration and repair after military-related muscle injury

Hypothesis: Angiotensin II receptor blockade will improve muscle healing after injury.

4) Progress to date (3-3-06 to 2-28-10):

a) Results of Original Technical Objective:

It was unknown whether suramin could improve muscle healing after contusion injury, the most commonly encountered muscle injury. Past studies had been performed on mice that had received laceration and strain injuries It also remains unclear whether this enhanced muscle regeneration is a direct effect of suramin. We have performed studies during the first year of funding to examine whether suramin would promote differentiation of myogenic cells *in vitro* and improve injured muscle healing by enhancing regeneration and reducing fibrosis *in vivo*, by using an animal model of muscle contusion.

Muscle-derived stem cell differentiation assay: Muscle-derived stem cells (MDSCs) were isolated from wild type mice (C57BL/6J) via the modified preplate technique. MDSCs (10^4 cells/well) were seeded into 12-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 10% horse serum, 0.5% chicken embryo extract, and 1% penicillin/streptomycin. After 24 hours, the medium was replaced with differentiation medium (DMEM containing 2% horse serum and 1% penicillin/streptomycin) containing different concentrations of suramin (0, 1, 10, and 100 μL/mL). After another 24 hours, the medium was replaced with differentiation medium. All cells were grown at 37°C in 5% CO_2. Three days after incubation, the fusion index was assessed by counting the number of nuclei in differentiated myotubes as a percentage of the total number of nuclei.

Immunocytochemistry in vitro: Immunocytochemistry was performed on the cells *in vitro* to examine their expression of fast myosin heavy chain (MyHC).

Animal model: The muscle contusion model was developed in normal wild-type mice (7 to10 weeks of age, with an average weight of 24.0g). A 17g stainless steel ball was dropped through an impactor from a height of 100 cm onto the animal's tibialis anterior (TA) muscle. Mice were divided into 4 groups (5 mice/group). Different concentrations of suramin (0, 2.5, 5, and 10 mg in 20 μL of phosphate-buffered saline [PBS]) were injected intramuscularly two weeks after injury. Statistical analysis was performed with ANOVA.

Suramin stimulates MDSC differentiation: Suramin treatment promoted the differentiation of MDSCs *in vitro* in a dose-dependent manner. We observed a significantly higher fusion index in each of the two suramin treatment groups (10 and 100 μg/mL**)** than in the control group (0 μg/mL). Furthermore, 100 μg/mL of suramin treatment enhanced the differentiation significantly more than the other suramin treatments (1 and 10 μg/mL).

Suramin enhances muscle regeneration and decreases fibrosis after contusion injury: We observed a significant increase in the number of regenerating myofibers in all of suramin treated groups (2.5, 5, and 10 mg/20 μL PBS) when compared with the control group (0mg/20μL of PBS).

Moreover, Masson's trichrome staining showed significantly less fibrotic area in all of suramin treated groups than in the control group. Although all three suramin treated groups showed significant improvement in healing by way of the enhancement of muscle regeneration and fibrosis inhibition, there was no significant difference between the three suramin treatment groups.

This data indicate that the suramin-treated MDSC groups have higher fusion indices than the control group *in vitro.* This suggests that suramin can enhance the differentiation of MDSCs, and reveals a portion of the mechanism by which suramin enhances muscle regeneration following injury. This is the first study to show that suramin not only has antiproliferative effects on fibroblasts, it affects the differentiation of MDSCs directly. Our results indicate that suramin can enhance muscle regeneration and prevent fibrosis after a contusion injury, the most common muscle injury **(Refer t o Appendices 1, 6 and 7)**. Our future study will investigate the mechanism(s) by which suramin enhances the differentiation of myogenic cells.

We have recently furthered these findings by performing a series of experiments to determine the mechanism (s) behind the beneficial effect of suramin on muscle healing after injury. Our hypothesis was that suramin enhances muscle healing by both stimulating muscle regeneration and preventing fibrosis in contused skeletal muscle. *In vitro*: Myoblasts (C2C12 cells) and muscle-derived stem cells (MDSCs) were cultured with suramin and the potential of suramin to induce their differentiation was evaluated. Furthermore, MDSCs were co-

cultured with suramin and myostatin (MSTN) to monitor the capability of suramin to neutralize the effect of MSTN. *In vivo*: Varying concentrations of suramin were injected in the tibialis anterior muscle of mice two weeks after muscle contusion injury. Muscle regeneration and scar tissue formation were evaluated by histological analysis and functional recovery was measured by physiological testing Our results demonstrated that suramin stimulated the differentiation of myoblasts and MDSCs in a dose-dependent manner. Moreover, suramin neutralized the inhibitory effect of MSTN on MDSC differentiation. *In vivo*, suramin treatment significantly promoted muscle regeneration, decreased fibrosis formation, reduced myostatin expression in injured muscle, and increased muscle strength after contusion injury.
In conclusion our results indicate that intramuscular injection of suramin following a contusion injury improved overall skeletal muscle healing. Suramin enhanced myoblast and MDSC differentiation and neutralized MSTN's negative effect on myogenic differentiation *in vitro*, which suggests a possible mechanism for the beneficial effects that this pharmacological agent exhibits *in vivo*. We believe that these findings could contribute to the development of biological treatments to aid in muscle healing after experiencing a muscle injury. These results were recently published in the American Journal of Sports Medicine (**refer to Appendix 7**).

In a final set of experiments, we have examined whether suramin treatment enhances muscle regeneration and reduce fibrosis by down-regulating myostatin expression *in vivo*. The muscle contusion was made on the tibialis anterior (TA) muscle of each mouse. Two weeks after injury, different concentrations of suramin (0 and 2.5 mg) were injected intramuscularly (n=20 mice/ group). At different time points (0.5, 1, 2, 10, and 14 days after injection), mice were sacrificed and cryosections of TA muscle were analyzed histologically. Suramin (2.5 mg) injection demonstrated a significant increase in the number of regenerating myofibers and reduction of fibrotic area when compared with the control group (0 mg). Furthermore, suramin injection effectively inhibited the expression of myostatin in the injured muscle. Our results suggest that suramin improves skeletal muscle healing by enhancing regeneration and reducing fibrosis after contusion injury through a potential decrease in myostatin expression in the injured skeletal muscle. Our findings may contribute to the development of progressive therapies for muscle injury. (**Refer to Appendices 1, 6 and 7)**

b) Results of Supplemental Proposals 1 and 2:

We have shown that decorin, a small leucine-rich proteoglycan, can inhibit TGF-β1 to prevent fibrous scar formation and improve muscle healing after injury. In the decorin-treated muscle, an enhancement of muscle regeneration is observed through histological examination. We have recently determined whether decorin has a direct effect on myogenic cells' differentiation. Our results indicate that myoblasts genetically engineered to express decorin (CD cells) differentiated into myotubes at a significantly higher rate than did control myoblasts (C2C12). This enhanced differentiation led to the up-regulation of myogenic genes (*Myf5*, *Myf6*, *MyoD*, and *myogenin*) in CD cells *in vitro*. We speculate that the higher rate of differentiation exhibited by the CD cells is due to the up-regulation of follistatin, PGC-1α, p21, and the myogenic genes, and the down-regulation of TGF-β1 and myostatin. Decorin gene transfer *in vivo* promoted skeletal muscle regeneration and accelerated muscle healing after injury. These results suggest that decorin not only prevents fibrosis, but also improves muscle regeneration and repair.

Recent studies have shown that myostatin (MSTN), first identified as a negative regulator of skeletal muscle growth, may also be involved in the formation of fibrosis within skeletal muscle. In a recent study, we further explored the potential fibrotic role of MSTN, as well as its interactions with both transforming growth factor-beta1 (TGF-β1) and decorin. We discovered that MSTN stimulated fibroblast proliferation in vitro, and induced its differentiation into myofibroblasts. We further found that, while TGF-β1 stimulated MSTN expression, MSTN stimulated TGF-β1 secretion in C2C12 myoblasts. Decorin, a small leucine-rich proteoglycan, was

found to neutralize the effects of MSTN in both fibroblasts and myoblasts, and up-regulate follistatin (FSTN), an antagonist of MSTN. Moreover, FSTN, an antagonist of MSTN, was up-regulated by decorin. The results of in vivo experiments showed that MSTN-knockout mice developed significantly less fibrosis and displayed better skeletal muscle regeneration when compared to wild-type mice at 2 and 4 weeks following laceration injury. In wild-type mice, we found that MSTN stimulated myofibers to express TGF-β1 in skeletal muscles at early time points following injection. Both TGF-β1 and MSTN were additionally seen to co-localize in myofibers in the early stages of injury. In summary, these findings define a fibrogenic property of MSTN, and indicate a coregulatory relationship between TGF-β1, MSTN, and decorin. Please **refer to Appendices 2, 3 and 4** . The complete recovery of injured skeletal muscle has posed a constant challenge for orthopaedic physician. Once injured, skeletal muscle is able to undergo regeneration from satellite cells; nevertheless, in the serious injured muscle, the formation of fibrosis often impedes effective muscle regeneration and resulted in an incomplete muscle healing. Therefore, to develop biological approaches to improve muscle healing, it is crucial to better understand the mechanisms of the skeletal muscle fibrosis. In the current studies, we found that myostatin (MSTN), a member of TGF-β family, plays a role in the formation of skeletal muscle fibrosis, besides the other putative fibrosis stimulator, TGF-β1. *In vitro*, MSTN directly stimulated the proliferation of fibroblasts and their productions of fibrotic proteins. *In vivo*, after laceration injury, gastrocnemius muscles of MSTN-/- mice showed less fibrosis and better muscle regeneration than wide-type (WT) counterparts. Considering MSTN as a therapeutic target of skeletal muscle healing, we found that inhibitors of MSTN, MSTN propeptide (MPRO) and follistatin, effectively blocked MSTN signaling and improved skeletal muscle healing after injured. We used adeno-associated virus (AAV)-mediated MPRO cDNA to successfully deliver MPRO *in vivo* and improve skeletal muscle healing of normal mice after laceration, and ameliorate dystrophic pathology of *mdx*/SCID mice. Furthermore, our results demonstrated FLST overexpression (FLST/OE) mice exhibited decreased fibrosis and increased muscle regeneration in injured skeletal muscle as compared to wild-type (WT) mice. Moreover, muscle progenitor cells (MPCs) isolated from MSTN-/- and FLST/OE mice significantly regenerated more myofibers than MPCs obtained from WT mice, when transplanted into dystrophic muscles. Collectively, our results suggested that MSTN directly stimulated fibrosis in the injured skeletal muscle; blocking MSTN signaling with MPRO or FLST improved skeletal muscle healing after laceration injury; blocking MSTN signaling in donor MPCs significantly enhanced the success of cell transplantation into dystrophic muscles. Our studies not only uncover some of the mechanisms implicated in skeletal muscle fibrosis and regeneration and help the development of new therapeutic approach for promoting the healing of injured or diseased skeletal muscle, but also renders a new sight of how to obtain robust genetically modified cell populations for cell therapy (**Refer to Appendices 5, 10, 12, 16 and 17**).

Interaction Between Macrophages, TGF-b1, and the COX-2 Pathway During the Inflammatory Phase of Skeletal Muscle Healing After Injury:

An important phase of skeletal muscle healing, largely involves macrophages, TGF-β1, and the COX-2 pathway. To improve our understanding of how these molecules interact during all phases of muscle healing, we examined their roles in muscle cells *in vitro* and *in vivo*. Initially, we found that depletion of macrophages in muscle tissue led to reduced muscle regeneration. Macrophages may influence healing by inducing the production of TGF-β1 and PGE2 in different muscle cell types. We then found that the addition of TGF-β1 induced PGE2 production in muscle cells, an effect probably mediated by COX-2 enzyme. It was also found that TGF-β1 enhanced macrophage infiltration in wild-type mice after muscle injury. However, this effect was not observed in COX-2_-/-_ mice, suggesting that the effect of TGF-β1 on macrophage infiltration is mediated by the COX-2 pathway. Furthermore, we found that PGE2 can inhibit the expression of TGF-β1. PGE2 and TGF-β1 may be involved in a negative feedback loop balancing the level of fibrosis formation during skeletal muscle healing. In conclusion, our results suggest a complex regulatory mechanism of skeletal muscle healing. Macrophages, TGF-β1, and the COX-2 pathway products may regulate one another's levels and have profound influence on the whole muscle healing process **(Appendix 9).**

Follistatin Improves Skeletal Muscle Healing by Blocking TGF-β-like Signaling Pathway:

Recovery from skeletal muscle injury is often incomplete due to inadequate myofiber regeneration and the formation of fibrosis. Accordingly, injured muscle can benefit significantly from therapies that stimulate muscle regeneration and inhibit fibrosis. To this end, we have focused on doing so by antagonizing a member of the TGF-β superfamily, myostatin. Myostatin is a pharmaceutical target that can be antagonized by follistatin. In vivo, follistatin over-expressing transgenic mice underwent significantly more myofiber regeneration and less fibrosis compared to wild type (WT) mice, as noted after skeletal muscle injury; this is likely partially because follistatin blocks myostatin activity and enhances vacularization. Additionally, the transplantation of muscle progenitor cells, isolated from follistatin over-expressing mice and WT mice, into the skeletal muscle of mdx/SCID mice revealed that the follistatin over-expressing donor cells are significantly superior to WT cells at regenerating skeletal muscle fibers. In vitro, follistatin stimulates myoblasts to express MyoD, Myf5, and myogenin, which are myogenic regulatory factors that promote the myogenic differentiation of myoblasts into myotubes. Furthermore, follistatin induces this enhanced differentiation through the inhibition of myostatin, activin A and transforming growth factor –beta 1, which are negative regulators of myoblast differentiation. This study suggests that follistatin is a promising agent for improving skeletal muscle healing; prior to its pharmacologic application; however, further investigation on follistatin is warranted (**Appendices 5 and 16**).

AAV-Mediated Myostatin Propeptide Gene Transfer Improved the Healing of Laceration Injured Skeletal Muscle and Muscle Progenitor Cell Transplantation Efficiency:

The development of approaches to improve muscle healing after injuries has focused largely on inhibiting fibrosis and promoting myogenesis. Since the inhibition of myostatin (MSTN), a negative regulator of skeletal muscle, causes a remarkable increase in skeletal muscle mass, we posited that MSTN blockade—with MSTN propeptide (MPRO) could improve muscle healing after injury. Our results demonstrated that the injection of an adeno-associated viral (AAV) MPRO vector into normal skeletal muscle, 4 weeks prior to creating a laceration injury in the muscle, led to an improvement of muscle healing when analyzed 4 weeks post-laceration and compared to the control groups. This effect was also observed in long term experimental animals which were sacrificed 1 year post-laceration. Next we explored the potential mechanisms by which MSTN blockade improved skeletal muscle healing. I*n vitro* we observed an enhancement of myoblasts' ability to differentiate into myotubes after AAV-2-MPRO gene transfer. We also demonstrated that muscle progenitor cells (MPCs) isolated from MSTN -/- mice regenerated significantly more myofibers than MPCs isolated from wild type (WT) mice when injected into the dystrophic skeletal muscle of mdx mice. Our results also suggest that MPROs beneficial effects are related to an increase in muscle regeneration, a reduction in fibrosis deposition and an increase in capillary in-growth into the injury site. These results suggest that MSTN blockade has a beneficial effect on muscle healing through an enhancement of the myogenic potential of MPCs as well as through the amelioration of the local environment within the injured skeletal muscle **(Appendices 10, 12 and 17).**

Muscle injuries are very common musculoskeletal problems encountered in sports medicine. Although these injuries are capable of healing, complete functional recovery is hindered by the formation of dense scar tissue triggered by TGF-β1. We have previously reported that several agents such as decorin and suramin which can inhibit fibrosis and improve regeneration in injured skeletal muscle; however, the safety of these agents remains unknown for treating muscle injury. By contrast, Losartan (LOS), one of the Angiotensin II Receptor Blockers (ARBs)—is an FDA approved antihypertensive medication and has been shown to also be antifibrotic in a variety of tissues, including skeletal muscle. This ARB has a well-tolerated side-effect profile, can also block TGF-β1 to attenuate the development of pathological fibrosis. In this study, we investigated optimum doses of LOS for treating injured muscle to help the translation of this research from bench to bedside.

The biphasic effect of LOS on C2C12 myoblasts *in vitro,* stimulating at low dose while decreasing at high dose, suggested that there is an optimal dose of LOS. Consequently we found that LOS improves skeletal muscle regeneration at 4 weeks after contusion injury, except that these effects were reduce/eliminated in 1000 mg/kg/day group. The best effective dose was 300 mg/kg/day. Overall, these effects of LOS were more

pronounced for regeneration than for fibrosis. These *in vivo* results are consistent with our *in vitro* results that LOS was able to exert effects on C2C12 whereas fibroblasts were not affected by either ANG or LOS. Regeneration and fibrosis are two competitive processes after muscle injury; therefore, decreasing fibrosis observed in LOS-treated group is can be the result of the increase regeneration. In other words, LOS might indirectly reduced fibrosis by directly stimulating regeneration. Although there were not statistical differences in fibrosis in lower dose LOS groups (3 and 30 mg/kg/day) as compared to control, these effects of LOS on both regeneration and fibrosis showed similar dose dependent trend. However, *in vivo* results above were obtained only from single time point after injury. Since there are time lags between peaks of myofiber regeneration and fibrosis after injury, further investigations are required to examine the effect of LOS on regeneration and fibrosis at their individual peak time, which will facilitate clinical application of ARBs in improving skeletal muscle healing (**Refer to Appendices 8 and 11**).

Angiotensin Receptor Blocker Improves Skeletal Muscle Functional Recovery in a Dose Dependent Manner:

We have previously reported that Losartan (LOS), one of the Angiotensin II Receptor Blockers (ARBs), which is FDA approved for antihypertensive treatment, has been shown to improve muscle healing through antifibrotic action [Bedair HS, *et al*., *Am J Sport Med*. 2008; 36: 1548-54]. We also demonstrated that specific doses of LOS (30 mg/kg/day and higher) improved muscle regeneration and attenuated the development of pathological fibrosis when it was administrated immediately after injury [Uehara K, *et al*., 55th Annual Meeting of the Orthopaedic Research Society, 2009]. In this study, we investigated whether LOS can improve muscle strength recovery after contusion injury, and also attempted to understand the mechanism of LOS action by analyzing gene expression of myostatin and follistatin, which are considered important regulators of skeletal muscle growth.

Improvement of Muscle Strength: LOS improved TA muscle force recovery after contusion injury. Specific peak twitch force and tetanic force was elevated in mice receiving high dose of LOS (30 and 300 mg/kg/day) in comparison to the animals receiving low dose of LOS (3 and 10 mg/kg/day).

Myostatin gene expression: Expression of myostatin in the injured TA muscle in the control-injury and low dose of LOS treatment groups was lower than in the normal TA muscle, while there was no difference between normal and high dose of LOS treatment groups. Expression of follistatin in the control group was higher than in normal muscle and low dose of LOS treatment groups. The highest expression of follistatin was observed in the 30 mg/kg/day of LOS treatment group and it was significantly higher compared to the control group.

Functional recovery is the most important factor in the skeletal muscle healing after injury. Here we demonstrate that LOS administration immediately after injury improves recovery of skeletal muscle strength. These results also support our previous histological findings [Uehara K, *et al*., 55th Annual Meeting of the Orthopaedic Research Society, 2009]. We believe that the mechanism of muscle regeneration after injury might be related to the expression of follistatin, positive regulator of skeletal muscle growth. We noticed over expression of follistatin in the 30 mg/kg/day treatment group compared to the normal and control-injury groups. These findings correlate with the results of physiological testing. It is unclear why the 30 mg/kg/day LOS displayed an increased expression of myostatin which is a negative regulator of skeletal muscle growth. Our results suggest that continuous administration of the high dose of LOS, in particularly 30 mg/kg/day, immediately after skeletal muscle injury could accelerate skeletal muscle functional recovery. We aimed to evaluate only a single time point of LOS administration immediately after injury. Further studies are required to determine the biological effect of LOS and facilitate the clinical application of ARBs for improvement of skeletal muscle healing **(Appendix 13).**

Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing:

Our previous study revealed that 30 mg/kg/day of LOS treatment was effective in promoting muscle healing and inducing an antifibrotic effect in a murine model of skeletal muscle after injury [Bedair HS, *et al*., *Am J Sport Med*. 2008; 36: 1548-54; Uehara K, *et al*., 55th Annual Meeting of the Orthopaedic Research

Society, 2009] . However, the effective dose (30 mg/kg/day) which was administrated immediately after muscle injury is higher compared to the dose used in humans (10 mg/kg/day). In this study we investigated the effect of muscle healing in a murine animal model using the recommended human dose of LOS (10 mg/kg/day) administered at different time points after injury.

LOS enhanced muscle regeneration and reduced fibrosis: We observed significant increases in the number of centronucleated myofibers in the day 3 treatment group when compared with other treatment groups The highest effect on muscle regeneration coincided with significant decrease of fibrosis in the day 3 treatment group.

LOS enhanced muscle force: LOS improved muscle strength recovery after contusion injury. Specific peak twitch force and peak tetanic force ws significantly greater in mice treated with LOS beginning at day 3 after injury.

LOS enhanced expression of Follistatin and Myostatin: Expression of FSTN detected by RT-PCR in the day 3 and day 14 LOS treatment groups was greater than in the normal non-injured or LOS treated at day 0 and day 7 groups. Expression of MSTN in the day 3, day 7 and day 14 LOS treatment groups was lower than in the normal, control and day 0 LOS treatment groups. The highest expression of FSTN coincided with the lowest expression of MSTN in the day 3 LOS treatment group.

This study revealed that the most effective timing for administration of human dose of LOS (10 mg/kg/day) was 3 days after muscle injury. We observed an increased number of centronucleated myofibers and decreased area of fibrosis when LOS was administered at day 3 after injury. The functional recovery after skeletal muscle injury is the most important factor for clinical translation of this therapy. We demonstrate that an enhancement of muscle strength in the day 3 LOS treatment group correlates with the improvement of muscle regeneration and the reduction of fibrosis. We have previously reported that muscle regeneration and fibrosis formation are two concomitant processes after muscle injury, and the effect of LOS was more prominent on muscle regeneration than on fibrosis [Uehara K, *et al*., 55th Annual Meeting of the Orthopaedic Research Society, 2009]. This current study supports this finding. In addition, it may suggest that the administration of LOS effectively leads to enhanced muscle regeneration after muscle injury via the down regulation of endogenous MSTN, which is a negative regulator of skeletal muscle growth. In summary, we indicate that 10 mg/kg/day (human safety dose) of LOS treatment initiated at 3 days after contusion injury can enhance structural and functional healing in mouse skeletal muscle. (**Appendix 14**)

Improving Recovery Following Recurrent Hamstring Injury Using an Angiotensin II Receptor Blocker:
Two Case Studies:

Note that the latter findings are provided only as translational evidence of the research being performed at the SCRC. The following studies were funded by the department of Orthopaedic Surgery at the University of Pittsburgh. Losartan was given to the patient volunteers as an off label use of the drug. No DOD funding was used for these studies.

Given that Losartan has already been used clinically with an extremelysafe side effect profile, physicians at the University of Pittsburgh's Department of Orthopaedic Surgery have conducted two case studies in young college athletes that sustained recurrent hamstring injuries and whose recoveries were safely improved with losartan. This is an off-label use of losartan (i.e.: the FDA has not approved labeling the device for the described purpose). Here we report the results obtained.

Subjects: Subject #1: male, 21 years old, college athlete (football punter). He presented 10 days after an acute onset of "searing" pain in his left posterior thigh when he was kicking with his left leg. He referred a similar injury 5 weeks prior to the present injury. Subject #2: male, 22 years old, college athlete (Ultimate Frisbee). He presented 4 days after an acute onset of pain in his left posterior thigh while he was sprinting. He referred two previous hamstring injuries (2 and 7 months prior to the present injury).

MRI results (at time of injury): Subject #1: Acute Grade 2 hamstring strain was observed with a partial thickness tear of the biceps femoris at the proximal myotendinous junction with surrounding edema without an

associated avulsion fracture or hematoma. Subject #2: Grade 2 strain with partial thickness tear of the left biceps femoris at the mid aspect, extends approximately 6 cm in the craniocaudal dimension.

Hamstring flexibility and strength: Subject #1: By the third week after the injury, no deficit was evident in hamstring flexibility. By the ninth week, the isometric hamstring strength measurements at 30 and 90 degrees of knee flexion were 92 and 84% than the uninjured side respectively (Fig.1). Subject #2: Also, by the third week after the injury, no deficit was evident in hamstring flexibility. By the ninth week, the injured side had a higher isometric hamstring strength measurement at 30 and 90 degrees of knee flexion. They were 132% and 110% than the uninjured side respectively.

We have described use of losartan, which is an FDA-approved angiotensin II receptor blocker, to treat two healthy collegiate athletes with a grade 2 biceps femoris injury. The patients tolerated the course of losartan well with no hypotension or any other side effects. Additionally, the patients demonstrated recovery of normal flexibility and strength compared to the contra-lateral leg. Both subjects were ready for return to sports in 9 to 11 weeks after injury (**Appendices 15 and 18**).

Key Research Accomplishment

- Determined that Suramin can stimulate MDSC myogenic differentiation *in vitro.*
- Determined that Suramin can enhance skeletal muscle regeneration and reduce fibrous scar formation following a contusion injury.
- Determined that Suramin's beneficial effects on muscle healing is related to its ability to enhance the proliferation and differentiation of myoblasts and MDSC and its ability to inhibit myostatin and up-regulate follistatin.
- Determined that the antifibrotic ability of decorin not only relates to its ability to inhibit TGF-β1 but also myostatin. We further showed that the up or down regulation of TGF-β1 simultaneously had the same effect on myostatin and vice versa.
- Demonstrated that macrophages, TGF- β1, and the COX-2 pathway products may regulate one another's levels and have profound influence on the whole muscle healing process.
- Myostatin has been shown to be a negative regulator of skeletal muscle regeneration and a positive regulator of fibrosis deposition which can be down-regulated with decorin and suramin
- Follistaitin is intricately involved with the muscle healing process and has been shown to be a positive regulator of muscle regeneration and a negative regulator of fibrosis deposition and can be up-regulated with decorin and suramin.
- Losartan has been shown to block TGF-β1 and have a statistically significant effect on the healing of injured skeletal muscle.
- Losartan is a safe and effective, clinically applicable angiotensin II receptor blocker and has been shown to be a potential safe and effective treatment for aiding in the healing of skeletal muscle injury.
- 1st case report of treating a recurrent hamstring injury in a 21 year old man with Losatrin is being prepared.

- The continuous administration of Losartan at markedly high dose of 30 mg/kg/day, immediately after skeletal muscle injury, can accelerate skeletal muscle functional recovery.

- 10 mg/kg/day (human safety dose) of LOS treatment initiated at 3 days after contusion injury can enhance structural and functional healing in mouse skeletal muscle.

- Follistatin stimulates myoblasts to express MyoD, Myf5, and myogenin, which are myogenic regulatory factors that promote the myogenic differentiation of myoblasts into myotubes.

- Follistatin induces differentiation through the inhibition of myostatin, activin A and transforming growth factor – beta 1, which are negative regulators of myoblast differentiation.

- MSTN blockade via MPRO has a beneficial effect on muscle healing through an enhancement of the myogenic potential of MPCs as well as through the amelioration of the local environment within the injured skeletal muscle.

- MPRO's beneficial effects are related to an increase in muscle regeneration, a reduction in fibrosis deposition and an increase in capillary in-growth into the injury site.

- Losartan was used to treat two healthy collegiate athletes with grade 2 biceps femoris injuries and tolerated its off-label use well with no hypotension or any other side effects. The patients demonstrated recovery of normal flexibility and strength compared to the contra-lateral leg and were capable of returning to sports activity in 9 to 11 weeks after injury.

Reportable Outcomes

1) **Nozaki M, Li Y, Zhu J, Ambrosio F, Fu FH and Huard J.** The use of suramin to improve skeletal muscle healing after contusion injury. Presented as a poster at the 53rd Annual Meeting; San Diego, CA; Feb 12-14, 2007. **(Appendix 1)**

2) **Li Y, Li J, Zhu J, Sun B, Branca M, Tang Y, Foster W, Xiao X, Huard J**. Decorin gene transfer promotes muscle cell differentiation and muscle regeneration. Mol Ther 2007 Sep; 15(9):1616-1622. **(Appendix 2)**

3) **Zhu J, Li Y, Shen W, Qiao C, Ambrosio F, Lavasani M, Nozaki M, Branca M, Huard J.** Relationships between TGF-β1, myostatin, and decorin: Implications for skeletal muscle fibrosis. J Biol Chem 2007 Aug; 282(35):25852-63. **(Appendix 3)**

4) **Zhu J, Li Y, Huard J**. Decorin Interacts with Myostatin Activity-Implications for Skeletal Muscle Healing. The Orthopaedic Research Society (ORS) 53rd Annual Meeting; San Diego, CA; Feb 12-14, 2007. **(Appendix 4)**

5) **Zhu J, Li Y, Branca M, Huard J**. Follistatin improves skeletal muscle healing after injury. The Orthopaedic Research Society (ORS) 53rd Annual Meeting; San Diego, CA; Feb 12-14, 2007. **(Appendix 5)**

6) **Shen W, Huard J.** Tissue therapy: Implications of regenerative medicine for skeletal muscle. Attala A, Lanza R, Thomson J, Nerem RM, editors. Principles of Regenerative Medicine. Elsevier Academic Press, 2007; 1232--1247, chapter 72. (Invited Book Chapter)

7) **Matsuura T, Li Y, Giacobino JP, Fu F, Huard J.** Skeletal muscle fiber type conversion during the repair of mouse soleus: Potential implications for muscle healing after injury. J. Orthop Res 2007 Nov; 25(11): 1534-1540**.** Figure featured on front cover as cover art.

8) **Nozaki M, Li Y, Zhu J, Uehara K, Ambrosio F, Fu F, Huard J.** Suramin can enhance the skeletal muscle healing by blocking myostation. The Orthopaedic Research Society (ORS) 54th Annual Meeting; March 2-5, 2008; San Francisco, CA. **(Appendix 6)**

9) **Nozaki M, Li Y, Zhu J, Ambrosio F, Uehara K, Fu F, Huard J.** Improved muscle healing after contusion injury by the inhibitory effect of Suramin on Myostatin, a negative regulator of muscle growth. Am J Sports Med, 2008 Dec; 36(12):2354-62. **(Appendix 7)**

10) **Bedair H, Karthikeyan T, Quintero AJ, Li Y, Huard J.** Angiotensin II Receptor Blockade administered after injury improves muscle regeneration and decreases fibrosis in normal skeletal muscle. Am J Sports Med, 2008 Aug; (36)8: 1548-1555. **(Appendix 8)**

11) **Shen W, Li Y, Zhu J, Schwendener R, Huard J.** Interaction between macrophages, TGF-beta 1, and the COX-2 pathway during the inflammatory phase of skeletal healing after injury. J of Cellular Physiology 2008 Feb; 214(2): 405-412. **(Appendix 9)**

12) **Ambrosio, F, Li Y, Usas A, Boninger, ML, Huard J**. Muscle repair after injury and disease. Orthopaedic Biology and Medicine, Musculoskeletal Tissue Regeneration: Biological Materials and Methods. The Humana Press Inc, 2008. (Invited Review)

13) **Zhu J, Ma J, Lu A, Qiao C, Li J, Li Y, Xiao X, Huard J**. Blocking Myostatin by AAV2-Delivered Myostatin Propeptide Improves Muscle Cell Transplantation **.** Orthopeadic Research Society; Las Vegas, Nevada; February 22-25, 2009. **(Appendix 10)**

14) **Uehara K, Nozaki M, Zhu J, Quintero A, Ota S, Fu F, Huard J. Angiotensin II Receptor Blocker Ameliorates** Skeletal Muscle Healing in a Dose Dependent Manner. 55th Annual Orthopeadic Research Society Meeting; Las Vegas, Nevada; February 22-25, 2009. **(Appendix 11)**

15) **Zhu J, Ma J, Qiao C, Jianbin L, Yong L, Xiao X, Huard J**. Blocking Myostatin Improves Muscle Healing Via Enhancement of Angiogenesis. 55th Annual Orthopeadic Research Society Meeting; Las Vegas, Nevada; February 22-25, 2009. **(Appendix 12)**

16) **Uehara, K; Kobayashi, T; Ota, S; Bin, S; Tobita, K; Ambrosio, F; Fu, FH; Huard, J**. Angiotensin Receptor Blocker Improves Skeletal Muscle Function Recovery in a Dose Dependent Manner. 56th Annual Orthopeadic Research Society Meeting; New Orleans, LA; March 6-9, 2010. **(Appendix 13)**

17) **Kobayashi, T; Uehara, K; Ota, S; Bin, S; Tobita, K; Ambrosio, F; Fu, FH; Huard, J.** Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing, 56th Annual Orthopeadic Research Society Meeting; New Orleans, LA; March 6-9, 2010.**(Appendix 14)**

18) **Yuri Chun MD, Sheila J M Ingham MD, James Irrgang PhD, Tanya Hagen MD, Freddie Fu MD, Burhan Gharaibeh, Vonda Wright MD, Johnny Huard, PhD.** Improving Recovery Following Recurrent Hamstring Injury Using an Angiotensin II Receptor Blocker: Two Case Studies; 56th Annual Orthopeadic Research Society Meeting; New Orleans, LA; March 6-9, 2010. **(Appendix 15)**

19) **Jinhong Zhu, M.D., Ph.D., Yong Li, M.D., Ph.D., Aiping Lu, MD., Burhan Gharaibeh, Ph.D., Jianqun Ma, M.D., Ph.D., Andres J. Quintero, M.D., Tetsuo Kobayashi, M.D., Johnny Huard, Ph.D.,** Follistatin Improves Skeletal Muscle Healing by Blocking TGF-β-like Signaling Pathway. **(In submission to Am J of Pathology, Appendix 16)**

20) **Jinhong Zhu, M.D., Ph.D., Jianqun Ma, M.D., Ph.D., Chunping Qiao, M.D., Jianbin Li, M.D., Andres J. Quintero, M.D., Yuri Chun, M.D., Aiping Lu, MD., Burhan Gharaibeh, Ph.D., Yong Li, M.D., Ph.D., Xiao Xiao, M.D., Ph.D., Johnny Huard, Ph.D.** AAV-Mediated Myostatin Propeptide Gene Transfer Improved the Healing of Laceration Injured Skeletal Muscle and Muscle Progenitor Cell Transplantation Efficiency. (**Under preparation for submission to Am J of Pathology, Appendix 17**)

1) **Kenji Uehara MD, PhD, Yuri Chun MD, Shusuke Ota MD, PhD, Tetsuo Kobayashi MD, PhD, Sheila J M. Ingham MD, Masahiro Nozaki MD, PhD, Kimimasa Tobita MD, James J. Irrgang PT, PhD, Freddie H. Fu MD, Vonda Wright MD, Tanya Hagen MD, and Johnny Huard PhD.** Improving Recovery Following Muscle Injury Using an Angiotensin II Receptor Blocker in both an animal model and in human patients: from the bench to the bedside. **(Under preparation for submission to Am J of Sports Medicine, Appendix 18)**

Conclusions

This line of work has enabled us to identify three potentially useful compounds that could be utilized to prevent fibrous scar formation and aid in the healing and regeneration of injured skeletal muscle. Suramin is a drug that has been used clinically to treat individuals infected with trypanosomes and worms and is currently being investigated for the treatment of prostate cancer; therefore it could potentially be applied clinically and expeditiously. Decorin too has shown great promise; however, it is not currently FDA approved for clinical application and would require more time to apply clinically since it would need to undergo clinical trials. From a basic science standpoint these studies have demonstrated the mechanisms that these compounds utilize for their beneficial effect on the healing and regeneration of skeletal muscle. They firstly act by inhibiting two components that are major initiators of the fibrosis cascade, TGF- β1 and myostatin. They have also been shown to facilitate regeneration and healing by promoting myoblast and MDSC proliferation and differentiation into myotubes in vitro and myofibers in vivo. One of the pathways that they seem to work on for this enhancement in regeneration appears to be the up-regulation of follistatin. Additional work is currently underway to determine the pharmokinetics of these two promising compounds. We have been exploring the use of the angiotensin II receptor blocker, Losartan, and have reported a case study that follows 2 patients and demonstrates that this drug was well tolerated by these 2 patients who were suffering from recurrent hamstring injuries. Additional animal studies are underway to explore the molecular mechanisms involved with the successful use of this drug to treat skeletal muscle injuries besides its ability to block TGF-β1. We have also been performing experiments with the myostain propepdtide (MPRO) which blocks the action of myostatin by directly binding to the molecule and hence inhibiting the fibrosis cascade from initiating. We have also shown that MPRO has a muscle regenerative action as well which has been shown to be related to the stimulation of angiogenesis in the injured muscle. Finally, we have explored the use of follistatin to inhibit fibrosis formation and increase muscle regeneration in injured skeletal muscle. We demonstrated that follistain blocks 3 major negative regulators of muscle growth including myostatin, TGF-beta1 and activin A. We also showed that follistatin can stimulate the myogenic differentiation of myoblasts which promotes muscle regeneration.

Appendices

Appendix 1: Nozaki M, Li Y, Zhu J, Ambrosio F, Fu FH and Huard J. The use of suramin to improve skeletal muscle healing after contusion injury. Presented as a poster at the Orthopaedic Research Society meeting, Feb. 2007.

Appendix 2: Li Y, Li J, Zhu J, Sun B, Branca M, Tang Y, Foster W, Xiao X, Huard J. Decorin gene transfer promotes muscle cell differentiation and muscle regeneration. Mol Ther 2007 Sep; 15(9):1616-1622.

Appendix 3: Zhu J, Li Y, Shen W, Qiao C, Ambrosio F, Lavasani M, Nozaki M, Branca M, Huard J. Relationships between TGF-β1, myostatin, and decorin: Implications for skeletal muscle fibrosis. J Biol Chem 2007 Aug; 282(35):25852-63.

Appendix 4: Zhu J, Li Y, Huard J. Decorin Interacts with Myostatin Activity-Implications for Skeletal Muscle Healing. The Orthopaedic Research Society (ORS) 53rd Annual Meeting; San Diego, CA; Feb 12-14, 2007.

Appendix 5: Zhu J, Li Y, Branca M, Huard J. Follistatin improves skeletal muscle healing after injury. The Orthopaedic Research Society (ORS) 53rd Annual Meeting; San Diego, CA; Feb 12-14, 2007.

Appendix 6: Nozaki M, Li Y, Zhu J, Uehara K, Ambrosio F, Fu F, Huard J. Suramin can enhance the skeletal muscle healing by blocking myostation. The Orthopaedic Research Society (ORS) 54th Annual Meeting; San Francisco, CA; March 2-5, 2008.

Appendix 7: Nozaki M, Li Y, Zhu J, Ambrosio F, Uehara K, Fu F, Huard J. Improved muscle healing after contusion injury by the inhibitory effect of Suramin on Myostatin, a negative regulator of muscle growth. Am J Sports Med, 2008 Dec; 36(12):2354-62.

Appendix 8: Bedair H, Karthikeyan T, Quintero AJ, Li Y, Huard J. Angiotensin II Receptor Blockade administered after injury improves muscle regeneration and decreases fibrosis in normal skeletal muscle. Am J Sports Med, 2008 Aug; (36)8: 1548-1555.

Appendix 9: Shen W, Li Y, Zhu J, Schwendener R, Huard J. Interaction between macrophages, TGF-beta 1, and the COX-2 pathway during the inflammatory phase of skeletal healing after injury. J of Cellular Physiology 2008 Feb; 214(2): 405-412.

Appendix 10: Zhu J, Ma J, Lu A, Qiao C, Li J, Li Y, Xiao X, Huard J. Blocking Myostatin by AAV2-Delivered Myostatin Propeptide Improves Muscle Cell Transplantation**.** Orthopeadic Research Society; Las Vegas, Nevada; February 22-25, 2009.

Appendix 11: Uehara K, Nozaki M, Zhu J, Quintero A, Ota S, Fu F, Huard J. Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing in a Dose Dependent Manner. Orthopeadic Research Society; Las Vegas, Nevada; February 22-25, 2009.

Appendix 12: Zhu J, Ma J, Qiao C, Jianbin L, Yong L, Xiao X, Huard J. Blocking Myostatin Improves Muscle Healing Via Enhancement of Angiogenesis. Orthopeadic Research Society; Las Vegas, Nevada; February 22-25, 2009.

Appendix 13: Uehara, K; Kobayashi, T; Ota, S; Bin, S; Tobita, K; Ambrosio, F; Fu, FH; Huard, J. Angiotensin Receptor Blocker Improves Skeletal Muscle Function Recovery in a Dose Dependent Manner. 56th Annual Orthopeadic Research Society Meeting; New Orleans, LA; March 6-9, 2010.

Appendix 14: Kobayashi, T; Uehara, K; Ota, S; Bin, S; Tobita, K; Ambrosio, F; Fu, FH; Huard, J. Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing, 56th Annual Orthopeadic Research Society Meeting; New Orleans, LA; March 6-9, 2010.

Appendix 15: Yuri Chun MD, Sheila J M Ingham MD, James Irrgang PhD, Tanya Hagen MD, Freddie Fu MD, Burhan Gharaibeh, Vonda Wright MD, Johnny Huard, PhD. Improving Recovery Following Recurrent Hamstring Injury Using an Angiotensin II Receptor Blocker: Two Case Studies; 56th Annual Orthopeadic Research Society Meeting; New Orleans, LA; March 6-9, 2010.

Appendix 16: Jinhong Zhu, M.D., Ph.D., Yong Li, M.D., Ph.D., Aiping Lu, MD., Burhan Gharaibeh, Ph.D., Jianqun Ma, M.D., Ph.D., Andres J. Quintero, M.D., Tetsuo Kobayashi, M.D., Johnny Huard, Ph.D., Follistatin Improves Skeletal Muscle Healing by Blocking TGF-β-like Signaling Pathway (**In submission to the Am J of Pathology**)

Appendix 17: Jinhong Zhu, M.D., Ph.D., Jianqun Ma, M.D., Ph.D., Chunping Qiao, M.D., Jianbin Li, M.D., Andres J. Quintero, M.D., Yuri Chun, M.D., Aiping Lu, MD., Burhan Gharaibeh, Ph.D., Yong Li, M.D., Ph.D., Xiao Xiao, M.D., Ph.D., Johnny Huard, Ph.D. AAV-Mediated Myostatin Propeptide Gene Transfer Improved the Healing of Laceration Injured Skeletal Muscle and Muscle Progenitor Cell Transplantation Efficiency. (**Under preparation for submission to the Am J of Pathology)**

Appendix 18: Kenji Uehara MD, PhD, Yuri Chun MD, Shusuke Ota MD, PhD, Tetsuo Kobayashi MD, PhD, Sheila J M. Ingham MD, Masahiro Nozaki MD, PhD, Kimimasa Tobita MD, James J. Irrgang PT, PhD, Freddie H. Fu MD, Vonda Wright MD, Tanya Hagen MD, and Johnny Huard PhD. Improving Recovery Following Muscle Injury Using an Angiotensin II Receptor Blocker in both an animal model and in human patients: from the bench to the bedside **(Under preparation for submission to the Am J of Sports Medicine)**

Project # 2
Improving muscle healing through digestion of scar tissue via MMP-1
(Yong Li)

Introduction:

Muscle injuries, which occur most frequently during sports activity, military training and battle; present a challenging problem in traumatology. After injury, damaged muscle fibers undergo a natural process of necrosis and the resulting dead tissue is removed by infiltrating lymphocytes. Meanwhile, locally released growth factors stimulate muscle regeneration by activating satellite cells. Unfortunately, the process of muscle regeneration is often incomplete from overgrowth of the extracellular matrix (ECM) leading to significant local fibrosis (i.e., fibrous scar formation). This scar tissue impedes the formation of normal muscle fibers in the injured muscle, resulting in incomplete functional recovery and a propensity for re-injury. We have begun to study the mechanism behind the fibrosis that occurs in injured skeletal muscle. Our previous studies have demonstrated that myogenic cells (including muscle-derived stem cells [MDSCs]) and regenerating myofibers in lacerated muscle can differentiate into fibrotic cells, and that transforming growth factor (TGF)-beta1 is a major stimulator of this differentiation. Using different animal models of muscle injury, we have investigated biological approaches by which to prevent fibrosis and thereby improve muscle healing. However, it often is not possible to treat injured muscles before the initiation of fibrosis—most patients with muscle injuries seek treatment only after the onset of fibrous scar formation, and the concomitant pain and functional deficits it produces. Moreover, chronic diseases (e.g., Duchenne muscular dystrophy [DMD]) generally present significant amounts of fibrous scar tissue within the patients' muscles. Because prevention of fibrosis is infeasible in many cases, the development of a novel therapeutic approach by which to digest existing fibrous scar tissue and improve muscle healing would be very significant.

Matrix metalloproteinase type-1 (MMP1), a naturally occurring collagen-digesting enzyme, has shown great capacity for digesting fibrous scar formations in various tissues. Additionally, MMP1 is also able to increase cell migration in many tissues. We believe that MMP1 is able to facilitate the healing of injured muscle by digesting fibrous scar tissue and improving the local environment in which muscle regeneration occurs. Results obtained from this project may lead to the development of gene therapy applications that eliminate scar tissue within skeletal muscle. Such applications could drastically improve the regeneration of muscles damaged by trauma or by chronic muscle diseases, such as Duchenne and Becker muscular dystrophies.

In the past few years, we have completed most of the proposed experiments. Through our work on **Objective #1**, we have determined that MMP1 increased muscle cell migration and differentiation/fusion capacity in vitro. In vivo, we also determined that MMP1 could improve muscle healing through increasing muscle cell migration, differentiation and regeneration (**Objective #2**). Finally, we have discovered that MMP1 gene transfer enhanced muscle cell migration and differentiation in vitro and in vivo (**Objective #3**).

Body:

Progress to date (3-3-06 to 2-28-10):

Technical Objective #1: To assess the effect of MMP1 on muscle cells *in vitro*.

This proposed experiment has been completed in the past two years. Results from this objective indicate that MMP1 is able to inhibit fibroblast growth and collagen deposition as well as enhance myoblasts migration and fusion/differentiation of these cells *in vitro*. The completed experiment has carried out two publications in the *Journal of Applied Physiology* [2007;102(6):2338-2345] and *American Journal of Pathology* [2009;174(2);541-549] and three review articles: *Future Medical Chemistry* [2009;1(6):1095-1111], *Cell Adhesion & Migration* [2009;3(4):337-341]. *US Musculoskeletal Review*, [2009; 4 (1):74-77].

Technical Objective #2: To assess the effects of MMP1 on skeletal muscle *in vivo*.

In this objective we observed the effects of MMP1 on skeletal muscle healing in animal models *(in vivo*). We have finished most of this proposed experiment. By using a reproducible muscle injury model in mice that induced significant fibrous scar formation, hindered myofiber regeneration and slowed muscle healing, we have determined that MMP1 can digest the existing fibrous scar and promote muscle healing in the injured skeletal muscles. This proposed experiment has been completed in the past year, and this study has been published by the *Journal of Applied Physiology* 2007;102(6):2338-2345. In addition, to observe the effect of MMP1 on muscle cell migration and differentiation in vivo, we have selected MDX mice, a mouse model for Duchenne muscular dystrophy [DMD], to investigate if MMP1 could enhance muscle cell migration and differentiation in vivo. Our results have substantiated this point and showed that MMP1 could enhance muscle cell fusion as well as migration in dystrophic muscle. This research have been published by *American Journal of Pathology* [2009;174(2);541-549] and have been discussed in our review papers: *Future Medical Chemistry* [2009;1(6):1095-1111], *Cell Adhesion & Migration* [2009;3(4):337-341]. *US Musculoskeletal Review*, [2009; 4 (1):74-77]. (Please see attached copy of manuscript).

Technical Objective #3: To assess the use of MMP1–based gene therapy to digest fibrous scar tissue within injured skeletal muscle.

The primary aim of this proposal is to investigate whether MMP1 gene transduction in implantable myoblasts can extend MMP1 function as a means of digesting fibrous scar tissue and promoting muscle cell migration, fusion and regeneration within injured skeletal muscle. MMP1 can degrade collagens, but undergoes self–degradation, which results in a short biological half-life. As a result, the fibrous scar tissue has the potential to reform following MMP1 degradation. To extend the function of MMP1, we have constructed a retrovirus vector encoding the MMP1 gene. These related studies and results are preparing one paper draft and is pending to send to publish. In the MMP1 gene therapy, we have discovered that #1. Human MMP1 gene expresses in target muscle cells; #2. MMP-1 gene transduction stimulated myoblasts to increase migration. #3. Gene transfer of MMP1 enhances myoblast differentiation *in vitro*. #4. MMP1 gene therapy improves myoblast transplantation in MDX/SCID (a dystrophic/immunodeficient mouse model) mice. #5. MMP1 gene transduction also increased myoblast migration after implantation *in vivo*. #6. Myoblasts genetically engineered to express MMP1 display a strong migration capacity following systemic delivery into *MDX/SCID* mice. These results support the notion that MMP1 is able to increase myoblast migration, differentiation and help injured skeletal muscle regeneration to speed healing process.

Future plans:

We have successfully completed all the proposed objectives. Our future plan will focus on the mechanism investigation of MMPs in muscle healing process and other potential function of MMP treatment in the musculoskeletal system which includes stem cell stimulation, angiogenesis and reinnervations in injured skeletal muscle.

Key Research Accomplishments:

1. Discovery of the effect of MMP1 on muscle cells, resulting in increased migration and differentiation of myogenic cells *in vitro*.
2. Discovery of the effect of MMP1 on scarring skeletal muscle, namely, the digestion of fibrous scar tissue, and improved muscle healing *in vivo*.
3. Discover of the effect of MMP1 on muscle cell migrations and fusion in skeletal muscle of mice *in vivo*.
4. Discovery of MMP1 gene transfer to extend its half-life, which prolongs MMP1 function during its application both *in vitro and in vivo*.

Reportable outcomes:

@ Four manuscripts:

1. Bedair H, Liu TT, Kaar J, Shown B, Russell A, Huard J, Li Y**.** Matrix Metalloproteinase (MMP) Therapy Improves Muscle Healing. ***J Applied Physiology*** 2007;102(6):2338-45.

2. Kaar JL, Li Y, Blair HC, Asche G, Koepsel RR, Huard J, Russell AJ. Matrix metalloproteinase-1 treatment of muscle fibrosis. ***Acta Biomater.*** 2008;4(5):1411-1420.

3. Wang W, Pan HY, Murray K, Jefferson M, Li Y. MMP1 promotes muscle cells migration and differentiation. ***American J Pathology*** 2009, 174 (2); 541-549.

4. Pan HY, Liu T, Wang B, Ikozawa M, Huard J, Li Y. MMP1 gene therapy to enhance muscle cell migration and differentiation. (in preparing)

@ Three review articles:

1. Mu XD, Wang W, Li Y. Myoblast transplantation and fibrosis prevention in diseased muscle (invited review). ***US Musculoskeletal Review*** 2009;4(1):74-77.

2. Bellayr I, Li Y. **Biochemical insights into the role of matrix metalloproteinases in the regeneration: challenges and recent developments.** Future Medicinal Chemistry 2009;1(6):1095-1111.

3**. Chen XP, Li Y. Role of matrix metalloproteinases in skeletal muscle: migration, differentiation, and regeneration.** ***Cell Adhesion & Migration*** 2009;3(4):337-341.

Conclusion:

In vitro, we have seen that MMP1 can enhance muscle cell migration and differentiation. The migration–related proteins are also up–regulated within MMP1–treated muscle cells. *In vivo*, the fibrous scar tissues that form within traumatically injured skeletal muscle could limit transplanted myogenic cell migration, fusion, and regeneration, thus slow the overall muscle healing process. However, muscle healing was greatly improved following MMP1 treatment within these scarred skeletal muscles. We also discovered that the use of MMP1 gene–transferred myoblasts resulted in an increased differentiation capacity *in vitro* and enhanced muscle cell migration *in vivo*.

Appendix and Reference:

1. Bedair H, Liu TT, Kaar J, Shown B, Russell A, Huard J, Li Y**.** Matrix Metalloproteinase (MMP) Therapy Improves Muscle Healing. ***J Applied Physiology*** 2007;102(6):2338-45.

2. Kaar JL, Li Y, Blair HC, Asche G, Koepsel RR, Huard J, Russell AJ. Matrix metalloproteinase-1 treatment of muscle fibrosis. ***Acta Biomater.*** 2008;4(5):1411-1420.

3. Wang W, Pan HY, Murray K, Jefferson M, Li Y. MMP1 promotes muscle cells migration and differentiation. ***American J Pathology*** 2009, 174 (2); 541-549.

4. Mu XD, Wang W, Li Y. Myoblast transplantation and fibrosis prevention in diseased muscle (invited review). ***US Musculoskeletal Review*** 2009;4(1):74-77.

5. Bellayr I, Li Y. **Biochemical insights into the role of matrix metalloproteinases in the regeneration: challenges and recent developments.** Future Medicinal Chemistry 2009;1(6):1095-1111.

6. Chen XP, Li Y. Role of matrix metalloproteinases in skeletal muscle: migration, differentiation, and regeneration. ***Cell Adhesion & Migration*** 2009;3(4):337-341.

7. Pan HY, Liu T, Wang B, Ikozawa M, Huard J, Li Y. MMP1 gene therapy to enhance muscle cell migration and differentiation. ***Molecule Therapy*** (in preparing)

Project # 3 Final Report**
Repairing injured skeletal muscle through myogenic endothelial cells
(Bruno Peault)

****Note that this subproject was completed last February 2009. Below is a reiteration of last year's final report**

INTRODUCTION

Upon muscle injury, satellite cells, the professional myogenic progenitors present in skeletal muscle, can divide, differentiate and fuse to generate myofibers [1, 2]. Injection of myoblasts has been attempted to repair both skeletal and cardiac muscles in animals and humans [3-5] and was to some extent successful but severe limitations were also encountered after myoblast implantation in both tissues[3, 6, 7,]. Muscle regeneration might be improved by transplanting stem cells, in some instances after therapeutic gene transfer, instead of later myogenic progenitors [8-12].

As described in other sections of this report, we have previously isolated, based on their slow adherence in culture, early myogenic progenitor cells, named muscle derived-stem cells (MDSC), that more efficiently regenerate both skeletal and cardiac muscles than satellite cells [9, 13]. MDSC were isolated retrospectively from cultured skeletal muscle; therefore their origin, identity and anatomic location in the native muscle are still unknown. The expression by MDSC of the myogenic cell markers desmin and MyoD, in addition to CD34 and Sca-1 and absence of c-kit and CD45, distinguishes these cells from other known adult stem cells such a MSC and MAPC.

We have, in the present study, investigated the identity and anatomical location of muscle-derived stem cells in human skeletal muscle. Our conclusion is that a previously unexplored developmental relationship exists between endothelial and myogenic cells. Indeed, a subset of MDSC express endothelial cell markers, and MDSC spontaneously differentiate into endothelial cells, and/or promote angiogenesis, probably *via* VEGF secretion, following implantation in skeletal and cardiac muscles [9, 13].

Besides regular satellite cells and endothelial cells, we have identified and characterized a novel population of *myo-endothelial* cells that co-express myogenic and endothelial cell markers. When injected into the injured skeletal muscles of immunodeficient mice, FACS-sorted cells co-expressing myogenic and endothelial cell markers (CD56+CD34+CD144+) regenerated skeletal muscle fibers much more efficiently than conventional CD56+ myogenic cells. Myoendothelial cells clonally expanded from single sorted cells differentiated into myogenic, chondrogenic and osteogenic cells under appropriate culture conditions.

KEY RESEARCH ACCOMPLISHMENTS

We have achieved the molecular identification and sorting of human myo-endothelial cells. Human skeletal muscle sections were immunostained with antibodies directed against myogenic and endothelial cell antigens. A subset of satellite cells (Pax7+ or CD56+) co-express endothelial cell antigens: VE-cadherin (CD144), vWF, the UEA-1 receptor and CD34. The percentages of Pax7+ cells also expressing these endothelial cell antigens were, respectively, 8.9%, 9.5%, 9.5% and 9.8%, as counted on at least 100 satellite cells in five independent experiments. Co-expression of these molecules by individual cells was ascertained by confocal microscopy. These results suggested the existence within adult human skeletal muscle of a rare subset of cells that co-express myogenic and endothelial cell markers. Adult skeletal muscle samples were then analyzed by flow cytometry after enzymatic dissociation. CD45- viable cells were gated and further separated into CD56+ myogenic cells and CD56- non-myogenic cells. As expected, the latter subset of non-myogenic cells contained the bulk of endothelial cells (CD34+CD144+). However, CD56+ cells also contained a very minor subset of cells expressing CD34 and CD144, thus confirming the existence of cells co-expressing markers of the myogenic and vascular endothelial cell lineages. Using the same immunostaining conditions, we then proceeded to sort the above-described cell subsets by FACS. The mean numbers of viable sorted cells which were recovered per experiment were $4.8 \pm 1.3 \times 10^4$ CD56+CD34-CD144- myogenic cells, $7.7 \pm 2.6 \times 10^4$ CD56-CD34+CD144+ endothelial cells and $1.5 \pm 0.8 \times 10^4$ CD56+CD34+CD144+ myoendothelial cells.

Purities of these three sorted cell populations were, respectively, 93.8 ± 1.0 %, 92.2 ± 1.1 % and 93.5 ± 1.8% [14-16]

We have regenerated skeletal muscle with human myoendothelial cells Myoendothelial, endothelial and satellite cells sorted from adult skeletal muscle were injected intramuscularly, in multiple distinct experiments, into SCID mouse skeletal muscles that had been injured by cardiotoxin. After 10 days, mice were sacrificed and skeletal muscles were analyzed by immunohistochemistry using an antibody directed to human spectrin. All three cell categories tested regenerated spectrin-expressing muscle fibers but quantitative analysis revealed that the regenerative potential of myoendothelial cells is by far the highest. Indeed, 1000 myoendothelial cells generated on average 89 myofibers, as compared with 9 and 5 myofibers produced when the same number of cells expressing exclusively endothelial or myogenic cell markers was injected, respectively. These results support our hypothesis that muscle vascular endothelial cells and a novel subset of cells with an overlapping phenotype between myogenic and endothelial cells are endowed with high muscle regeneration potential[14-16]

We have cultured myoendothelial cells over the long term, and shown that these proliferate faster, and survive better under oxidative stress than myogenic cells and endothelial cells Endothelial, myoendothelial and satellite cells were cultured independently in the medium used for MDSC culture[9] for 5 to 6 weeks. All 3 subsets of long-term cultured cells remained capable of differentiating into myotubes in vitro. Cultured cells injected into SCID mouse muscles damaged with cardiotoxin also retained their ability to regenerate myofibers, and myoendothelial cells remained the most efficient myogenic progenitors in these assays.

Myoendothelial cells proliferate significantly faster than the two other cell groups. Having previously observed that the high regenerative capacity of mouse MDSC in skeletal and cardiac muscles is related to their resistance to oxidative stress [13], we treated the three populations under study with hydrogen peroxide in culture. Myoendothelial cells are the most resistant to oxidative stress as indicated by lower levels of cell death under these conditions. [14-16]

We have shown that myoendothelial cells are, at the clonal level, multi-lineage mesodermal stem cells We investigated the multipotency of myoendothelial cells, isolated from adult human skeletal muscle, at the single-cell level. Myoendothelial cell clones express markers of myogenic, endothelial, perivascular and mesenchymal stem cells (MSCs). These clonal cells can be differentiated into endothelial cells, smooth muscle cells, pericytes, adipocytes, chondrocytes and osteoblasts *in vitro* and participated in vessel, bone and cartilage regeneration *in vivo*. We therefore demonstrate that human myoendothelial cells possess some fundamental properties of stem cells.[17]

REPORTABLE OUTCOMES

- Peault, B. et al. Stem and Progenitor Cells in Skeletal Muscle Development, Maintenance, and Therapy. *Mol Ther* (2007)
- Zheng, B., Cao, B., Crisan, M., Sun, B., Li, G.H., Logar, A., Yap, S., Pollett, J.B., Drowley, L., Cassino, T., Gharaibeh, B., Deasy, B., Huard, J., Péault, B. Prospective identification of myogenic endothelial cells in human skeletal muscle. *Nature Biotechnology*, 25(9):1025-1034 (2007
- Crisan, M., Deasy, B., Gavina, M., Zheng, B., Huard, J., Lazzari, L., Péault, B. Purification and long-term culture of multipotent progenitor cells affiliated with the walls of human blood vessels: myoendothelial cells and pericytes. *Methods In Cell Biology*, 86:295-309. (2008)
- Crisan, M., Zheng, B., Sun, B., Yap, S., Logar, A., Huard, J., Giacobino, J.P., Casteilla, L., Péault, B. Purification and culture of human blood vessel-associated progenitor cells. Current Methods in Stem Cell Biology. In Press.
- Zheng, B., Li,G., Deasy, B., Pollett. J., Sun, B, Drowley, L., Gharabeh, B., Usas, A., Logar, A, Peault, B., and Huard, J. The myoendothelial cell: a novel stem cell from adult human skeletal muscle. *In preparation*

Although the present project has been focused on the regeneration of *skeletal* muscle, we have also documented the strong potential of these cells to regenerate the myocardium and improve cardiac function post-infarction:

- Okada, M., Payne, T., Zheng, B., Oshima, H., Momoi, N., Tobita, K., Keller, B.B., Phillippi, J., Péault B., Huard, J. Improved cellular cardiomyoplasty via human myogenic-endothelial cells identification of a progenitor cell population from human skeletal muscle that is superior to committed skeletal myoblasts for cardiac cell therapy. *Journal of the American College of Cardiology*. (2008)

CONCLUSIONS

The experiments performed in this project have provided evidence for the existence of a strong myogenic potential in cells related to the endothelial cell lineage in the normal, human adult skeletal muscle, and therefore suggest a developmental relationship between vascular cells and myogenic cells. We have indeed confirmed the existence of a novel population of cells present between human muscle fibers that co-express the nuclear Pax7 myogenesis-specific transcription factor and surface antigens that typify endothelial cells: VE-cadherin (CD144) and vWF.

The leading conclusion of the present work is that human muscle-derived myoendothelial cells can regenerate skeletal muscle, quantitatively more efficiently than regular myogenic (satellite) cells. The higher regeneration capacity exhibited by myoendothelial cells is due in part to better resistance to oxidative stress, a likely environmental condition within the injured skeletal muscle. These data suggest the existence of a novel hierarchy within human adult skeletal muscle along which vascular endothelial cells give rise to early myogenic stem cells, which in turn replenish the satellite cell population.

These novel myogenic progenitors are amenable to biotechnological processing; these cells can be sorted to homogeneity by flow cytometry from skeletal muscle, then cultured clonally on the long term, with no major loss of developmental potential, in basic medium, where they proliferate rapidly but do not become tumorigenic. Therefore, the transplantation of such autologous endothelium-related progenitors could be envisioned shortly as a therapy of skeletal muscle diseases and injuries.

REFERENCES

1. Bischoff, R. Proliferation of muscle satellite cells on intact myofibers in culture. *Dev Biol* 115, 129-139 (1986).
2. Bischoff, R. in Myology: Basic and Clinical 97-118 (McGraw-Hll, New York; 1994).
3. Skuk, D. & Tremblay, J.P. Myoblast transplantation: the current status of a potential therapeutic tool for myopathies. *J Muscle Res Cell Motil* 24, 285-300 (2003).
4. Partridge, T.A. Invited review: myoblast transfer: a possible therapy for inherited myopathies? *Muscle Nerve* 14, 197-212 (1991).
5. Menasche, P. et al. Myoblast transplantation for heart failure. *Lancet* 357, 279-280 (2001).
6. Cao, B., Deasy, B.M., Pollett, J. & Huard, J. Cell therapy for muscle regeneration and repair. *Phys Med Rehabil Clin N Am* 16, 889-907, viii (2005).
7. Menasche, P. Cellular transplantation: hurdles remaining before widespread clinical use. *Curr Opin Cardiol* 19, 154-161 (2004).
8. Miller, J.B., Schaefer, L. & Dominov, J.A. Seeking muscle stem cells. *Curr Top Dev Biol* 43, 191-219 (1999).
9. Qu-Petersen, Z. et al. Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. *J Cell Biol* 157, 851-864 (2002).
10. Gussoni, E. et al. Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* 401, 390-394 (1999).

11. Partridge, T.A. Stem cell therapies for neuromuscular diseases. *Acta Neurol Belg* 104, 141-147 (2004).
12. Peault, B. et al. Stem and Progenitor Cells in Skeletal Muscle Development, Maintenance, and Therapy. *Mol Ther* (2007).
13. Oshima, H. et al. Differential myocardial infarct repair with muscle stem cells compared to myoblasts. *Mol Ther* 12, 1130-1141 (2005).
14. Zheng, B., Cao, B., Crisan, M., Sun, B., Li, G.H., Logar, A., Yap, S., Pollett, J.B., Drowley, L., Cassino, T., Gharaibeh, B., Deasy, B., Huard, J., Péault, B. Prospective identification of myogenic endothelial cells in human skeletal muscle. *Nature Biotechnology*, 25(9):1025-1034 (2007
15. Crisan, M., Deasy, B., Gavina, M., Zheng, B., Huard, J., Lazzari, L., Péault, B. Purification and long-term culture of multipotent progenitor cells affiliated with the walls of human blood vessels: myoendothelial cells and pericytes. *Methods In Cell Biology*, 86:295-309. (2008)
16. Crisan, M., Zheng, B., Sun, B., Yap, S., Logar, A., Huard, J., Giacobino, J.P., Casteilla, L., Péault, B. Purification and culture of human blood vessel-associated progenitor cells. Current Methods in Stem Cell Biology. In Press.
17. Zheng, B., Li,G., Deasy, B., Pollett. J., Sun, B, Drowley, L., Gharabeh, B., Usas, A., Logar, A, Peault, B., and Huard, J. The myoendothelial cell: a novel stem cell from adult human skeletal muscle. *In preparation*

Project # 4 Final Report **
Cell therapy for muscle regeneration advances via interdisciplinary-driven regenerative medicine (iDREAM)
(Bridget Deasy)

****Note that this subproject was completed last February 2009. Below is a reiteration of last year's final report**

Key Research Accomplishments

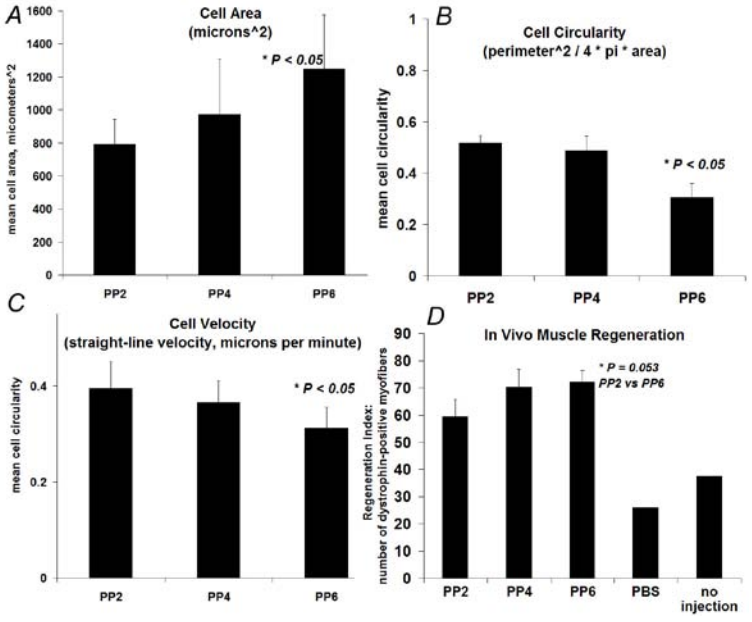
1. **Technical Objective #1:** Use preplate isolation technique and a bioinformatic cell culture system to screen adult human stem cell candidates for their muscle regeneration potential.
2. **Technical Objective #2:** Test culture conditions for extensive and long-term expansion of human myogenic cells to preserve the cells' phenotype and regeneration efficiency.

We have completed these aims. First we have identified that the most potent human muscle stem cell candidate can be isolated from slowly adhering fractions of the cell suspension which derives from the muscle biopsy. To identify these cells we first examined the cells via live cell imaging, and second we examined the cells in an in vivo model of muscular dystrophy.

Through a collaboration with Cook MyoSite Inc (Pittsburgh, PA), we received samples of human muscle cells. These cells were isolated based on their variable adherence, using a method similar to the preplate technique which was performed with mouse myogenic cells. It has been previously shown that the preplate isolation technique can be used to isolate stem cells from the skeletal muscle of mouse [32] and rat [75], while direct defined FACS-sorting has been performed using tissue from mouse [32], rat [75] and human sources[18]. Usually, myogenic precursors with characteristics of myoblasts or satellite cells are found within the early preplates (PP1–3), whereas cells isolated from the later preplates (PP5-6) have stem cell characteristics[1]. We term the cells PP2, PP4 and PP6-- PP2 adhere prior to PP4 which adhere prior to PP6.

These results provide examples that illustrate a relation between in vitro and in vivo characteristics. Human MDCs were analyzed for the presence of the cell surface cluster of differentiation markers CD34, CD56, CD144, and CD146 by flow cytometry. For live cell imaging analysis 3 separate hMDCs preplate populations were examined – PP2, PP4, and PP6. By the preplate method, PP2 corresponds to myoblasts and PP6 corresponds to the muscle-derived stem cell candidate[1,77,79]. The cells were imaged using the automated cell imaging system[81]. Three separate visible image sequences (20x) were captured at 10 minute intervals for each well over a culture time of 3 days. Image sequences were imported to ImageJ for user interactive analysis. Measurements included 17 parameters including area, centroid, center of mass, perimeter, circularity, skewness, and best fit ellipse. Sequences were divided into 18 hour increments with 10 separate cells outlined and measured over 30 minutes at each time point. Results from ImageJ measurement were imported into a predesigned worksheet for calculation and analysis. Cell motility was described as centroid velocity, dividing the change in centroid position by the scan time interval. Single cell measurements were averaged over each time point and image sequence yielding a

Figure 1



morphological description of each well for the culture period. The mean area for PP2 (792±151 um^2) was significantly smaller (P=0.022, type-2, 2-tailed) than PP6 (1248±327 um^2, **Fig 1A**). Furthermore, PP2 velocity (0.395±0.056 um/min) was significantly greater (P=0.031) than PP6 (0.312±0.044 um/min, **Fig 1B**). Finally, examination of circularity (a measure of cell roundness) showed PP2 (0.517±0.028) and PP4 (0.487±0.058) were significantly different from PP6 (0.307±0.052, P<0.01, **Fig 1C**). Flow cytometry showed all PPs were negative for CD34 & CD144, with decreasing expression for CD146 & CD56 over time in culture. In sum, the results show that PP2 cells are significantly smaller and more motile with greater circularity than PP6, two populations previously resolved only by adherence rates. The LCI approach shows the ability to resolve differences in cell populations based on a number of behavioral measures only a few of which are presented here. Moreover subsequent in vivo experiments showed that this in vitro difference correlates with an in vivo difference in performance (**Fig 1D**).

We have also expanded these cells under various cell culture conditions and we confirm that the phenotype of the cells is altered through standard culture conditions (**Fig 2**). We found that cell lose expression of the myogenic marker CD56 and the progenitor marker CD146 as they are expanded (**Fig 3**) as the are expanded.

It is worth noting that whereas we have expanded mouse MDSC populations to beyond 200 population doublings (PDs), our results to date show that human MDSC populations will reach senescence much sooner than mouse MDSCs when grown in the same growth medium of 20% serum Dulbecco's Modified Eagle Medium (DMEM). **Figure 2A** depicts these differences in the proliferation kinetics of human and mouse MDSCs (in 20% DMEM, 10^9 mouse MDSCs vs. 10^{12} human MDSCs, at 7 weeks, n=1). These results underscore the need to more extensively study growth factor-expansion methods for human stem cells. Using commercially available, chemically defined media, we were able to stimulate human muscle–derived stem cell expansion in the *in vitro* expansion assay (**Figure 2**). We have observed that other human muscle cells-myoendothelial cells-

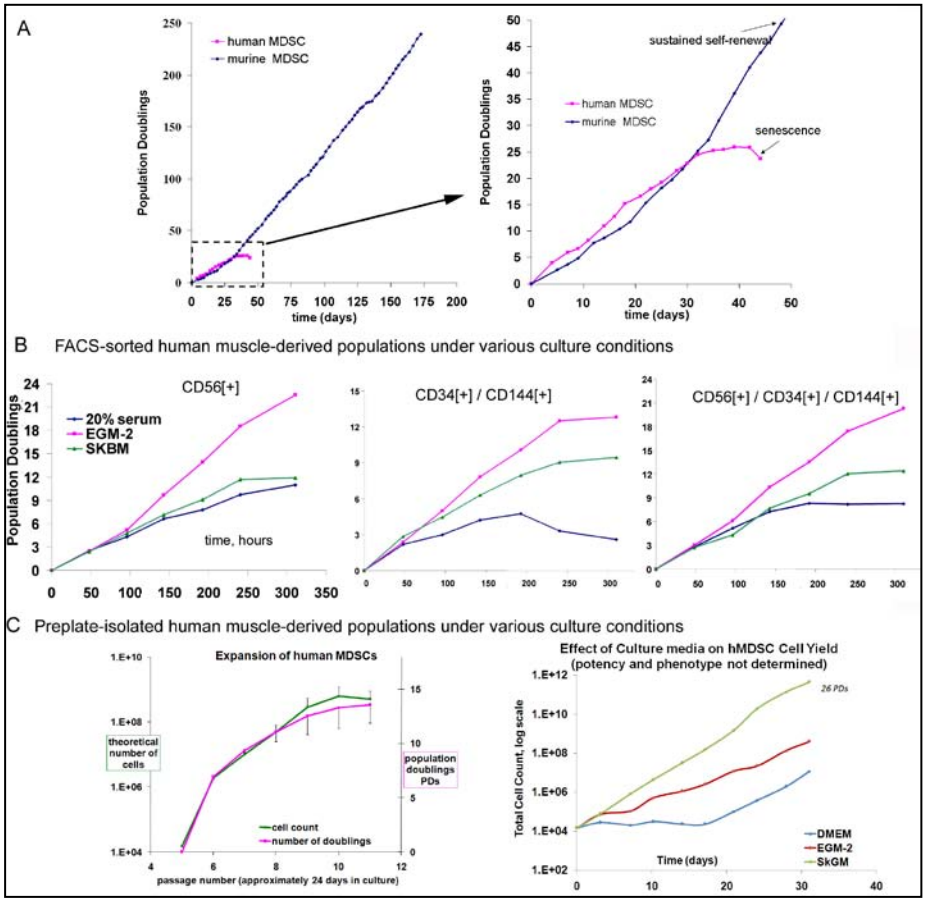


Figure 2. Human MDSC expansion. (A) hMDSC have slower proliferation rates as compared to mouse MDSC *Human myogenic cells were cultured using the same techniques used for mouse MDSCs; yet, a striking difference in their expansion potential was observed.* ***(B)*** *Expansion of FACS-isolated human myogenic progenitors was examined using various culture media (20% serum, EGM2 and SKGM). Medium supplemented with endothelial growth factor (EGM2) stimulated the greatest amount of expansion for all hMDCs. The results of panel (B) were published in Zheng et al 2007 Nat Biotech.* ***(C)*** *While hMDSC show promise for clinical treatment of urinary incontinence much work needs to be performed to expand these cells to higher cell doses to reach a much larger set of tissues to be effective for muscular dystrophies.*

have exhibited increased expansion when cultured in defined media such as endothelial growth medium 2 (EGM2, Clonetics) or skeletal basal medium (SKBM, Clonetics) ([56], **Figure 2B**). Expansion of FACS-isolated human myogenic progenitors was examined using 3 different culture media (20% serum, EGM2 and SKGM [56]). Three purified populations were examined – CD56[+] cells, CD34[+]CD144[+] cells and CD56[+] CD34[+] CD144[+] cells. All three purified human populations demonstrated the highest degree of expansion with EGM-2 media **(Figure 2B).**

The results shown in **Figure 2B** were recently included in a report by Zheng et al, in which the authors suggest that the MDSCs may have their developmental origins as myoendothelial cells[56,57]. Subsequently we performed another examination of the effect of 3 different culture media on the preplate derived human MDSC populations. In this case, we observed the greatest expansion potential with SKGM (**Figure 2**), and not EGM2 which favored human myoendothelial cells sorted by FACS. While this may suggest that the human myoendothelial cells and the human preplate MDSCs are unique populations, those experiments have not been performed side-by-side, and it is not our goal to compare these populations. Rather, these findings show that optimization of cell expansion is cell-line specific and the human preplate cells can be expanded to high numbers using SKGM as compared to EGM2 (**Figure 2**). We examined phenotype during the course of cell expansion. All 3 preplate fractions were initially negative for CD34 and CD144 (data not shown). The PP2, PP4, and PP6 fractions expressed significantly different amounts of the myogenic marker desmin (**Figure 3A**) with the PP2 (fast adhering) fraction containing the highest percentage of positive cells ($p < 0.05$ as compared to the slow adhering or PP6 fraction). We also observed variable levels of CD56 and CD146, and these markers decreased with cell passage. **Figure 3B** shows the changes in CD56 which occur as cells are cultured. The fast-adhering (PP2) fraction, and moderately adhering (PP4) fraction show a decrease in CD56 expression through the course of 3 cell passages (passage 7 through passage 9, **Figure 3B**). The slow (PP6) fraction maintains a low level of CD56 through the course of these passages. We have also examined the PP6 / hMDSCs fraction for expression of CD90, CD105 and CD73, which are expressed by mesenchymal stem cells. The populations are >90% positive for these markers (flow cytometry and PCR results, data not shown). These results and others suggest that hMDSCs may be obtained from the PP6 fraction which appears to be distinct from PP2.

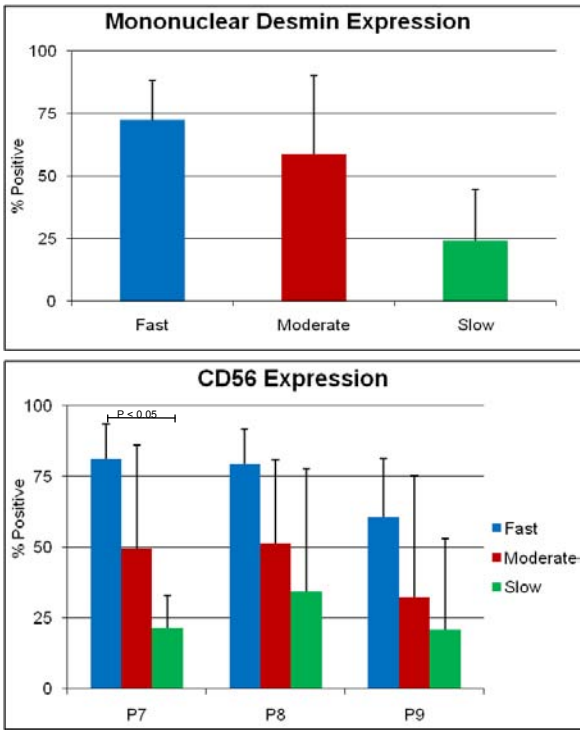


Figure 3. *Human preplate populations. PP2=Fast adhering fraction, PP4=moderate rate of adherence, PP6=slow adherence rate during the preplate isolation process. These populations differ in their expression of* ***(A)*** *desmin (via immunocytochemistry) and* ***(B)*** *CD56 (via flow cytometry). We also illustrate in (B) that the expression of this marker changes with cell expansion (passage 7-passage 9 are shown).*

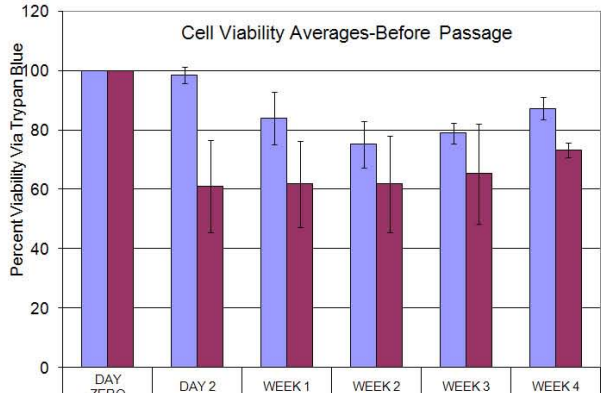
Supplemental Objectives

Technical Objective #1: Use a bioinformatic LCI culture system to test the effect of temperature on the p rocessing of MD SC f rom s keletal muscle biopsy. Here we will consider the various temperature cycles that the biopsy may undergo prior to stem cell isolation. We will examine differences in percent yield based on the length of time that a biopsy is at 4C storage. We will examine the phenotypes of MDSCs from various isolations using the LCI tools.

Progress: The instrumentation for these studies is now in place and we have initiated studies of the effects of temperature on stem cell viability. The delivery of the imaging system for these experiments was delivered on Jan 14, 2009 and calibration was complete on Jan 30, 2009. Therefore, it was necessary for us to request a no-cost extension on these objectives. We have performed initial experiments to examine cell viability at lower temperatures and to date we confirmed viability and cell proliferation, however, the complete analysis of the effect of temperature is still ongoing.

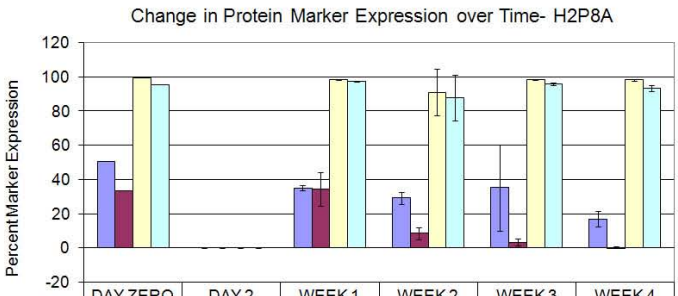
Technical O bjective # 2: T o test t he ef fect of various cyroprotectant agents and procedures on the stem cell phenotype of human MDSC. In this objective, we will obtain unique comprehensive phenotype profiles of muscle-derived stem cell populations after various preservation protocols. We will also examine various thawing procedures and test the percent cell recovery after thawing. Most important we will test the quality of the stem cells to perform in multilineage differentiation assays. We will use the optimal human MDSC phenotype (identified in DOD 2005-2007) to identify which processing procedures are optimized for maintaining stem cell quality and quantity.

A



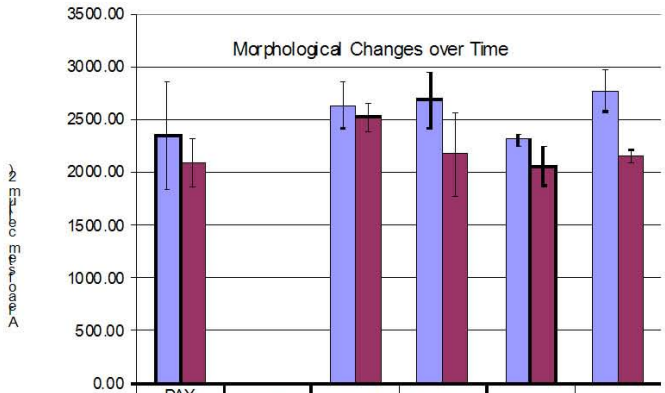
	DAY ZERO	DAY 2	WEEK 1	WEEK 2	WEEK 3	WEEK 4
H2 P8 A- 10% DMSO	100	98.41269841	84.01292335	75.13597514	78.93939394	87.24747475
H2 P8 B- 20% DMSO	100	61.02693603	61.81318681	61.80555556	65.27777778	73.21428571

B



	DAY ZERO	DAY 2	WEEK 1	WEEK 2	WEEK 3	WEEK 4
CD 56	50.57	0	34.94	29.2033333	35.1866666	16.9066666
CD 146	33.27	0	34.35	8.46666666	3.31	0.30333333
CD 73	99.36	0	98.38	90.95	98.3533333	98.16
CD 105	95.57	0	97.31	87.86	95.78	93.3266666

C



	DAY ZERO	DAY 2	WEEK 1	WEEK 2	WEEK 3	WEEK 4
H2P8 A- 10% DMSO	2357.32	0.00	2640.07	2685.01	2314.12	2775.48
H2P8B-20% DMSO	2090.50	0.00	2530.19	2174.56	2061.97	2155.19

Figure 4. We have already examined the effect of DMSO and gl ycerol on t he viability and st ability of another ce ll t ype w hich i s being us ing i n hu man clinical t rials for ur inary i ncontinence (muscle derived ce lls, M DCs, C ook M yosyte). I n t his collaborative study, we observe the best post-thaw recovery after freezing with 10% DMSO and compared to 20% DMSO (glycerol not shown)

Progress: We have identified the optimal MDSC population as presented above and we have examined several cryoprotectants agents to examine the effect of freezing on the phenotype and cell viability. Human muscle stem cells which may be used in a clinical setting for cell therapeutics will need to be harvested, processed in a GMP facility, and cryopreserved prior to use. Each of these steps will expose the cells to reagents which may damage the cells or induce phenotypic changes. In addition, each step may occur at a different temperature, and the stem cells are therefore exposed to temperature cycles which are potentially damaging.

We currently collaborate with a local biotech company CookMyosite which studies human muscle-derived cells for use in urinary incontinence. In fact this company is currently conducting clinical trials transplanting these cells to the urethra of patients with urinary incontinence. In the Deasy lab, we have studied the effect of cryopreservation on these cells. In these studies, human muscle stem cells were added to a solution of fetal bovine serum (FBS) and a cryoprotectant (DMSO or glycerol) at 100,000 cells per cryopreservation vial. We examined 6 timepoints (day 0, day 2, week 1-4), done in triplicate. At the given timepoints, cells are slow frozen in the -80°C freezer, then quick thawed in the room temperature waterbath. For each time point , cells are thawed from cryopreservation vials, counted for viability via Trypan Blue, put in culture for 24 hours, imaged in T-25 plates, then passaged, counted again for viability via Trypan Blue (**Fig.4a**) . Remaining cells are flowed through the flow cytometer to determine percent marker expression (**Fig.4b**). Images of cells in cultured in T-25 analyzed in Image J for morphological changes (**Fig.4c).**

Under the conditions studied here, we observe a moderate about of cell death which could be improved by using higher concentrations of DMSO. Slow freezing stem cells kills around 50% cells from day 0 to week 4. We observe that10% DMSO is a better concentration than 20%- provides increased cell viability. Thawed muscle cells lost their expression of protein markers CD56 and CD146. H2P9A exhibited a 67% decrease in CD56; 99% decrease in CD146. H2P9B exhibited a 68% decrease in CD56; 97% decrease in CD146. H2P8A cell area increased by 18%; H2P8B cell area increased by 3%. There also was a significant difference in the number of cells remaining at week 4 compared to day 0, and there was significant difference in the percent marker expression of CD56 and CD146 comparing day 0 to week 4 ($p < 0.05$). There is no significant difference in area between day 0 data and week 4 for both samples.

In this Phase I project, we will focus on the effect of cryopreservation on human umbilical cord derived cells. Currently, dimethyl sulfoxide, DMSO, is the cryopreservation reagent utilized in the preservation of these cells. The cells are normally frozen in a solution of 10% DMSO, and stored at -80 C or -196 C, for days, weeks, or months. For clinical applications, human MDSCs have been shipped in a frozen state from the processing facility to the clinic(*56, 116*). It is key that these cells retain their stem cell phenotype after thawing.

Recommended ch anges: In the original protocol, we proposed to examine the effect of temperature fluctuations of cell phenotype. We propose to continue with these aims. We also have no recommended changes to the study of cryopreservation of the human muscle-derived cells.

Publications which have resulted from this work:

1. Chirieleison SM, Schugar RC, Deasy BM. 2008. Current Progress in Cell-mediated Gene Therapy for Muscular Dystrophies. *Research Advances in Gene Therapy*. Global Research Network. Editor: RM Mohan.
2. Schmidt, BT, Feduska, JM, Witt, AM, and BM Deasy. 2008 Robotic cell culture system for stem cell assays. *Industrial Robot*. *35 (2):116-124.*

Abstracts that have resulted from this work :

1. Chirieleison SM , Scelfo CC, Askew Y, Deasy BM. 2008. Development of Morphological Measurement Scheme Using Open Source Software for Live Cell Imaging Approaches to Stem Cell Biology. University of Pittsburgh Science 2008; Pittsburgh, PA, USA. POSTER
2. Chirieleison SM , Scelfo CC, Deasy BM. 2008. Quantitative Analysis of Therapeutic Muscle Cell Populations through Live Automated Cell Imaging. American Society for Cell Biology Annual Meeting; San Francisco, CA, USA. POSTER
3. Chirieleison SM , Scelfo CC, Askew Y, Deasy BM. 2008. Development of Morphological Measurement Scheme Using Open Source Software for Live Cell Imaging Approaches to Stem Cell Biology. Pittsburgh Orthopaedic Journal; Pittsburgh, PA, USA.
4. Scelfo CC, Chirieleison SM , Deasy BM. 2008. Integration and Adaptation of Live Cell Image Analysis. Pittsburgh Orthopaedic Journal; Pittsburgh, PA, USA.

5. Chirieleison SM , JM Feduska, RC Schugar, SL Sanford, J Huard, BM Deasy. 2008. Identifying Populations of Human Muscle Derived Stem Cells to Participate in Skeletal Muscle Regeneration Based on Phenotypic Differences. Pittsburgh Orthopaedic Journal; Pittsburgh, PA, USA.
6. Chirieleison SM , Feduska JM, Schugar RC, Witt AM, Deasy BM. 2008. In Vitro Aging of Human Muscle Stem Cells Due to Culture Expansion. International Society for Stem Cell Research; Philadelphia, PA, USA. POSTER
7. Chirieleison SM , Scelfo, CC, Askew Y, Deasy BM. 2008. Development of Morphological Measurement Scheme Using Open Source Software for Live Cell Imaging Approaches to Stem Cell Biology. International Society for Stem Cell Research, Philadelphia, PA, USA. POSTER
8. Chirieleison SM , Feduska JM, Schugar RC, Sanford SL, Huard J, and Deasy BM. 2008. Identifying Regenerative Populations of Human Muscle-Derived Cells and Tracking Phenotypic Changes in Culture. MidWest Tissue Engineering Consortium. Cincinnati, OH, USA. PODIUM
9. Chirieleison SM , Feduska JM, Schugar RC, Sanford SL, Huard J, Deasy BM. 2008. Identifying Populations of Human Muscle Derived Stem Cells to Participate in Skeletal Muscle Regeneration Based on Phenotypic Differences. Orthopaedic Research Society; San Francisco, CA, USA. POSTER

Project # 5 Final Report**
Inhibiting cell death and promoting muscle growth for congenital muscular dystrophy
(Xiao Xiao)

****Note that this subproject was completed last February 2009. Below is a reiteration of last year's final report**

Introduction

Congenital muscular dystrophy (CMD) is a group of severe forms of muscular dystrophy leading to early death in human patients(1-3, 7). The majority of cases are caused by genetic mutations in the major laminin, laminin containing the α2 chain (formerly named merosin), in the muscle basement membrane(4, 6, 8, 11). The early morbidity/fatality and the lack of effective treatment require urgent search for novel therapeutics. Previously, we utilized mini-agrin, which has been proven to have a therapeutic effect in transgenic MCMD mice, to treat MCMD mice by AAV vector(10). Our preliminary studies showed that over-expression of mini-agrin protein greatly improved general health and muscle morphology in MCMD mice. However, the treated disease mice still developed gradual paralysis and displayed shorter life span than wild type mice. To further improve the current gene therapy paradigm, we have developed complementary gene therapy strategy for laminin alpha2-deficient CMD.

Body

As mentioned earlier, we utilized laminin alpha2 deficient congenital muscular dystrophy (MDC1A), a severe form of muscular dystrophy animal model, as our muscle injure model in this proposal. We evaluated the therapeutic effects of delivering different molecules, such as insulin-like growth factor 1 (IGF-1), anti-apoptotic gene BCL-XL, as well as myostatin propeptide, on muscle and nerve pathogenesis of MDC1A. While we did not observe the significant therapeutic effects by single delivery of those factors on MDC1A mice, we made very interesting discovery during those experiments and wrapped up two manuscripts. Since the first manuscript has been published {Qiao, 2008 #980}, we will mainly concentrate on the second manuscript in this report.

AAV vector transduced white matter of spinal cord

Previously, we have revealed that delivering AAV serotype 8 vectors to neonatal mice can efficiently transduce whole-body skeletal muscle and cardiac muscle. In the present study, we utilized the same AAV serotype and similar delivery route to investigate the ability of AAV vector to infect peripheral nervous system and spinal cord. For the first set of study, we utilized AAV8-CMV-LacZ vector. Two months after vector injection, the mice were sacrificed. The whole spine including bone and adjacent muscle were cut into three pieces and quick-frozen away. After cryo-section, the slides were subjected to LacZ staining, and H& E staining were

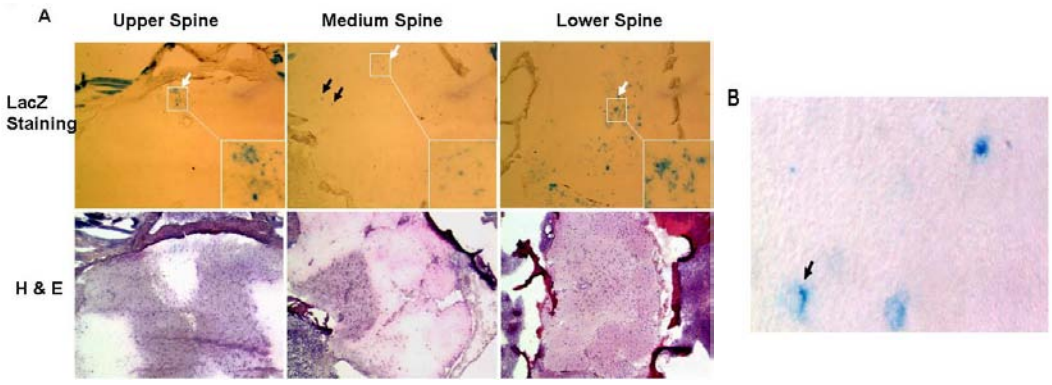


Figure 1. LacZ staining of spinal cord from AAV8-LacZ injected mice. A. LacZ positive cells in white matter. The vector was delivered on 3-day-old neonatal mice, and the mice were sacrificed at 2-month-old age. Notably, most of the lacZ positive spots were in white matter (white arrow), and very few LacZ positive cells were in grey matter (black arrow). Insets were indicated enlarged area. The consecutive sections of H&E staining were used to display the structure of spinal cord. It was also apparent that there were more LacZ positive cells in lower spine, followed by medium spine, and then upper spine. B. Few LacZ positive cells in grey matter. Button-like structure (black arrow) in grey matter indicated small neuron and axon communication.

performed on the consecutive sections to display the histology. As shown in figure 1A, most LacZ positive cells were located in white matter in the spinal cord of AAV8-LacZ delivered mice (white arrow and inset area). Occasionally, we could see few LacZ positive cells in grey matter (black arrow and figure 1B). Notably, there were more LacZ positive cells in the lower spine and less LacZ positive cels in the higher spine potion. Large magnification of few LacZ positive cells in grey matter of ventral horns showed button-like structures in close proximity and overlapping neuronal cells (Fig 1B). AAV8 vector effectively transduced dorsal root ganglion and peripheral nerves

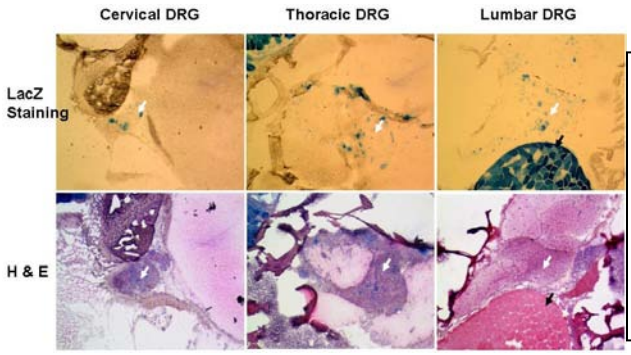


Figure 2. LacZ staining indicated AAV8-LacZ vector efficiently transduced dorsal root ganglion (DRG). White arrow indicated DRG, and the consecutive section of H&E staining were utilized to display the DRG structure. Black arrow pointed to adjacent LacZ positive muscle cells

In addition to the white matter, we noticed AAV8 vector could efficiently infect dorsal root ganglion (DRG) cells which were positioned outside of the spinal cord (Fig2 and Fig 3, white arrow). Across the whole spine, all the DRG neuronal cells were effectively transduced by AAV vector (Fig.2).

In the second set of our study, we wished to use a different report gene, such as GFP gene, to further prove our finding. Similar to LacZ vector, AAV8-D(+)-CMV-GFP vector was delivered into neonatal mice. Two months after vector injection, the mice were sacrificed. To reduce the background staining of spinal cord, we did perfusion to remove circulating blood and fixed with 4% paraformaldehyde (under anesthesia) when the mice were sacrificed. Figure 3 displayed middle portion of the spine, most of the GFP positive cells were located in while matter of spinal cord and DRG (white arrow).

Except for white matter of spinal cord and DRG, we also observed LacZ and GFP positive cells in peripheral nerve fibers (Fig 4).

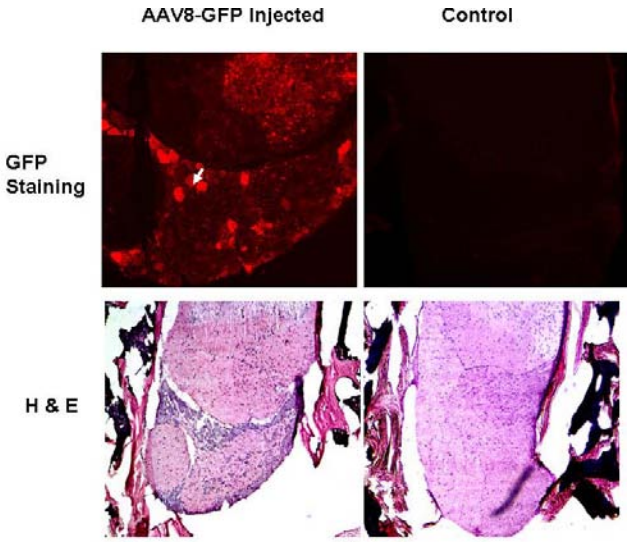


Figure 3. Immunofluorescent staining of GFP in spinal cord and dorsal root ganglion from AAV8-GFP delivered mice.

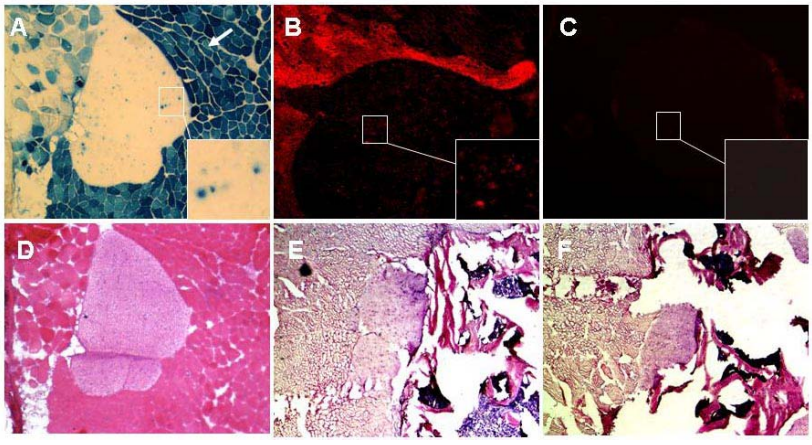


Figure 4. AAV vector efficiently transduced peripheral nerve roots. A was from AAV8-LacZ injected mice, and B was from AAV8-GFP injected mice, C was from mock injected animal but stained with GFP. D, E, F were consecutive section of H& E staining. Insects were indicated enlarged area of peripheral nerves. The LacZ positive cells surrounding peripheral nerves in picture A (white arrow) were muscle cells.

Key Research Accomplishments:

1. We have delivered myostatin propeptide gene into laminin alpha2-deficient CMD mice and the therapeutic effect was evaluated. Unlike mdx mice for which myostatin propeptide delivery can ameliorate its muscle pathology, the delivery of propeptide had a severe side effect on laminin alpha2-deficient CMD mice. The injected mice died even early than untreated controls. This phenomenon is consistent with previous discovery(5).
2. We have delivered AAV-IGF1 vector into laminin alpha2-deficient CMD mice and did not observe significant therapeutic effect.
3. We have delivered AAV-BCL-XL into laminin alpha2-deficient CMD mice and did not observe significant therapeutic effect.

Reportable Outcomes:

Our manuscript entitled "Myostatin propeptide gene delivery by AAV8 vectors enhances muscle growth and ameliorates dystrophic phenotypes in *mdx* mice" has been published in Human Gene Therapy 2008 Mar; 19(3): 241-54(9).

Our second manuscript titled "Efficient gene transfer to dorsal root ganglion and peripheral nerves via systemic delivery of AAV vectors" is in preparation and will be submitted soon.

Conclusions:

Single AAV-IGF1, AAV-Bcl-XL, or AAV-MPRO gene delivery can not offer therapeutic benefit for laminin alpha 2 deficient dy^w/dy^w mice.

AAV can efficiently transduce peripheral nervous system and dorsal root ganglion cells.

References

1. **Allamand, V., and P. Guicheney.** 2002. Merosin-deficient congenital muscular dystrophy, autosomal recessive (MDC1A, MIM#156225, LAMA2 gene coding for alpha2 chain of laminin). Eur J Hum Genet **10:**91-4.
2. **D'Alessandro, M., I. Naom, A. Ferlini, C. Sewry, V. Dubowitz, and F. Muntoni.** 1999. Is there selection in favour of heterozygotes in families with merosin-deficient congenital muscular dystrophy? Hum Genet **105:**308-13.
3. **Emery, A. E.** 2002. The muscular dystrophies. Lancet **359:**687-95.
4. **Kuang, W., H. Xu, P. H. Vachon, L. Liu, F. Loechel, U. M. Wewer, and E. Engvall.** 1998. Merosin-deficient congenital muscular dystrophy. Partial genetic correction in two mouse models. J Clin Invest **102:**844-52.
5. **Li, Z. F., G. D. Shelton, and E. Engvall.** 2005. Elimination of myostatin does not combat muscular dystrophy in dy mice but increases postnatal lethality. Am J Pathol **166:**491-7.
6. **O'Brien, D. P., G. C. Johnson, L. A. Liu, L. T. Guo, E. Engvall, H. C. Powell, and G. D. Shelton.** 2001. Laminin alpha 2 (merosin)-deficient muscular dystrophy and demyelinating neuropathy in two cats. J Neurol Sci **189:**37-43.
7. **Pegoraro, E., M. Fanin, C. P. Trevisan, C. Angelini, and E. P. Hoffman.** 2000. A novel laminin alpha2 isoform in severe laminin alpha2 deficient congenital muscular dystrophy. Neurology **55:**1128-34.
8. **Pegoraro, E., H. Marks, C. A. Garcia, T. Crawford, P. Mancias, A. M. Connolly, M. Fanin, F. Martinello, C. P. Trevisan, C. Angelini, A. Stella, M. Scavina, R. L. Munk, S. Servidei, C. C. Bonnemann, T. Bertorini, G. Acsadi, C. E. Thompson, D. Gagnon, G. Hoganson, V. Carver, R. A.**

Zimmerman, and E. P. Hoffman. 1998. Laminin alpha2 muscular dystrophy: genotype/phenotype studies of 22 patients. Neurology **51:**101-10.

9. **Qiao, C., J. Li, J. Jiang, X. Zhu, B. Wang, J. Li, and X. Xiao.** 2008. Myostatin Propeptide Gene Delivery by Adeno-Associated Virus Serotype 8 Vectors Enhances Muscle Growth and Ameliorates Dystrophic Phenotypes in mdx Mice. Hum Gene Ther **19:**241-54.

10. **Qiao, C., J. Li, T. Zhu, R. Draviam, S. Watkins, X. Ye, C. Chen, J. Li, and X. Xiao.** 2005. Amelioration of laminin-{alpha}2-deficient congenital muscular dystrophy by somatic gene transfer of miniagrin. Proc Natl Acad Sci U S A.

11. **Tome, F. M., T. Evangelista, A. Leclerc, Y. Sunada, E. Manole, B. Estournet, A. Barois, K. P. Campbell, and M. Fardeau.** 1994. Congenital muscular dystrophy with merosin deficiency. C R Acad Sci III **317:**351-7.

Project # 6 Final Report**
Treatment for Muscle Wasting
(Paula Clemens)

****Note that this subproject was completed last February 2009. Below is a reiteration of last year's final report**

Introduction:

The ability to promote muscle regeneration in the setting of focal or generalized muscle loss could confer significant clinical benefit in the setting of focal neuropathic or other processes that cause muscle atrophy or chronic illnesses that cause cachexia. We hypothesize that gene transfer strategies can promote muscle regeneration toward a goal of improving muscle bulk and strength in the setting of injuries or diseases that cause muscle atrophy.

Extensive evidence has shown that higher levels of some pro-inflammatory cytokines contribute to the development of cachexia. For example, serum tumor necrosis factor α (TNFα) and IL-1α levels are markedly increased in patients with rheumatoid arthritis and cancer. Other cytokines, such as IL-6, IL-1β and proteolysis-inducing factor (PIF) have been reported to contribute to the development of muscle wasting.

Elegant studies show that TNFα binds to its receptor on skeletal muscle resulting in the activation of nuclear factor κB (NF-κB) mediated by phosphorylation and degradation of the NF-κB inhibitory protein, IκBα. Downstream effects of pathological NF-κB activation in skeletal muscle include the inhibition of new muscle formation and the degeneration of existing muscle. *In vitro* studies support the potential that the IκBα superrepressor (IκBSR), an IκBα genetically engineered to prevent its phosphorylation, can prevent the activation of NF-κB in skeletal muscle and could ameliorate or prevent muscle wasting. Our preliminary studies demonstrate the novel determination of inhibition of activation of NF-κB by cellular FLIP (cFLIP).

Our hypothesis is that the inhibition of the downstream pathways of TNFα causing muscle atrophy and failure of muscle regeneration should be effected intracellularly in muscle fibers. A gene therapy strategy is ideal for the purpose of achieving an ongoing effect that is cell-type restricted. We anticipate that inhibition of NF-κB activation restricted to muscle fibers will provide a therapeutic effect on muscle while avoiding potential toxic effects of inhibiting NF-κB activation in other tissues.

Body:

To characterize an *in vitro* model of cancer-induced muscle wasting in primary muscle cells and in stable muscle cell lines expressing IκBSR or cFLIP.

We developed a new *in vitro* cell culture assay to study the effects of cancer cell cytokines on muscle cell differentiation and use this assay to test novel gene transfer approaches for the treatment of cancer cachexia. Exposure to conditioned media from selected human cancer cell lines resulted in failure of muscle cell differentiation. A known intracellular mechanism of NF-κB activation as a cause of cancer cachexia was recapitulated in this *in vitro* system. We observed a direct correlation between NF-κB activation and inhibition of myogenic differentiation in the *in vitro* assay. Exposure to inflammatory cytokines and to conditioned media from human cancer cells each resulted in NF-κB activation within primary muscle cells. Failure of myogenic differentiation and the associated activation of NF-κB were prevented by stable expression of either IKBSR or cFLIP, but not by Bcl-xL. These findings were published. As described below, we used the PC-3 cell line to generate a new *in vivo* cancer cachexia model in mice.

Taken together, this study provides an *in vitro* assay that demonstrates secretion of cachexia-inducing factors by certain cancer cell lines that result in the inhibition of myogenic differentiation by activation of NF-κB. Over-

expression of cFLIP in muscle cells inhibits both NF-κB-mediated and apoptotic pathways, thereby preventing tumor media-induced inhibition of myogenic differentiation and cytotoxicity. These findings point toward the potential to design novel molecular therapeutics for the treatment of cancer-induced muscle wasting.

To clone, rescue, and purify adeno-associated viral (AAV) vectors carrying IκBSR or cFLIP.
We are generating vectors with either ubiquitous or muscle-specific promoter control of expression. AAV serotype 8 vectors provide high levels of muscle transduction and the ability to transduce muscle by systemic delivery. Where possible, we are using self-complementary vectors that result in the highest possible levels of transgene expression.
At our last report, we had rescued the following vectors as high-titer, purified AAV8 vectors: Conventional single strand AAV8 vectors were rescued with the following expression cassettes: CMV-GFP and CMV-cFLIP. Self-complimentary (double strand) AAV8 vectors were rescued with the following expression cassettes: CMV-IκBSR and MCK-GFP. The double strand MCK-GFP vector has been tested by direct intramuscular injection showing high levels of transgene expression.

Since the last report, we rescued the MCK-IκBSR double strand vector as AAV8. The MCK-cFLIP double strand vector plasmid was difficult to clone, but we finally achieved it. We are currently rescuing that plasmid as AAV8. We observed widespread skeletal muscle expression from the MCK-GFP AAV8 vector after systemic delivery. We also observed skeletal muscle expression after intramuscular and systemic delivery of the MCK-IκBSR AAV8 vector.

Characterization of a new PC-3 cell cancer cachexia model in mice.
We have refined our model of muscle cachexia in mice in order to test molecular therapeutics *in vivo*. We generated a novel model of cancer cachexia induced by PC-3 cells and compared it to an established model induced by MCA-26 cells. We reported initial findings on the *in vivo* PC-3 cell cancer cachexia model in our last report. We have now completed those studies and prepared a manuscript for submission. We showed that tibialis anterior and quadriceps muscles had increased activation of NF-κB in the PC-3 cell model of cancer cachexia, as predicted from our *in vitro* studies exposing muscle cells in culture to supernatant from PC-3 cells. Interestingly, the gastrocnemius, which did not show an increase in activated NF-κB, instead showed increased levels of phosphorylated eukaryotic initiation factor 2 alpha (p-eIF2-α) indicating decreased protein synthesis contributing to the cachexia phenotype. This raises the interesting possibility that increased protein degradation or decreased protein synthesis may contribute to cachexia to different degrees in different muscle groups.

To apply AAV8 vector strategies to ameliorate cancer cachexia.
Five-week old CD2F1 mice received intramuscular administration of either AAV8-MCK-dsIκBSR or AAV8-CMV-dsIκBSR or AAV8-CMV-ss cFLIP vector 1 week prior to MCA-26 tumor inoculation. Body weight and tumor growth were monitored every other day. All mice developed tumors and cachexia beginning at about 12 days after tumor inoculation (Figure 1). Three weeks tumor after innoculation the mice were sacrificed and hind-limb muscles were dissected, weighed, and snap-frozen for analysis. The tibialis anterior muscle injected with AAV8-MCK-dsIκBSR showed a significant increase in weight compared to tumor-bearing control (Figure 2). Tibialis anterior muscle injected with each of the 3 therapeutic AAV8 vectors showed a significant increase in muscle fiber diameters compared to tumor-bearing control (Figure 3).

Our results suggest that vectors expressing proteins with the intended effect of decreasing NF-κB activation can ameliorate cancer cachexia *in vivo*. Future studies are needed to test vector-mediated gene delivery of IκBSR and cFLIP by systemic delivery in cancer cachexia models.

Figure 1A

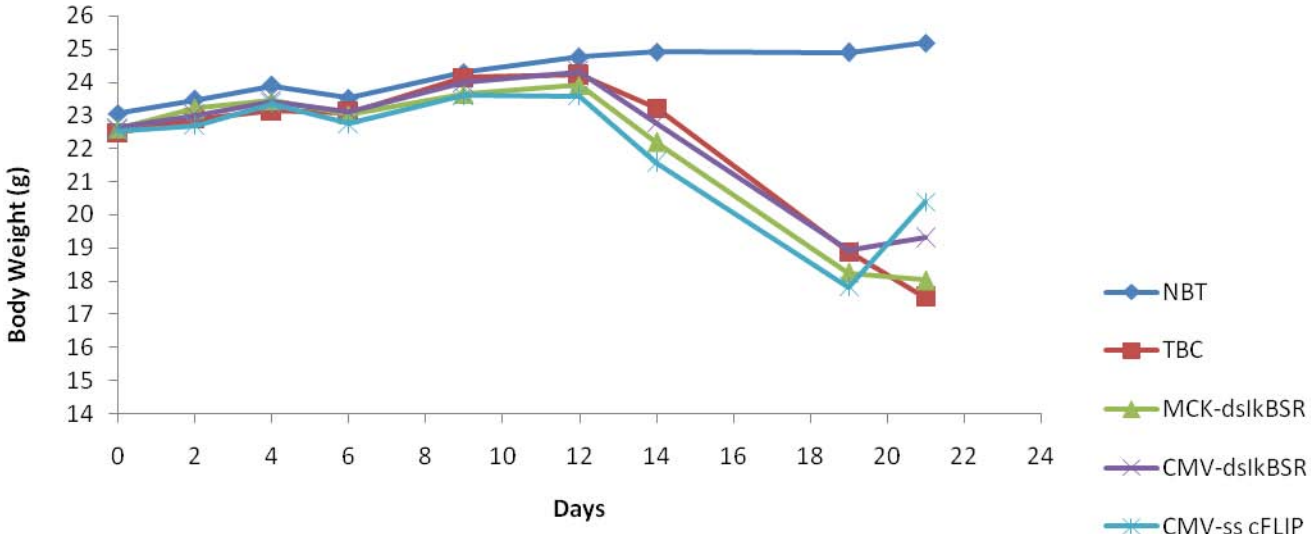


Figure 1B

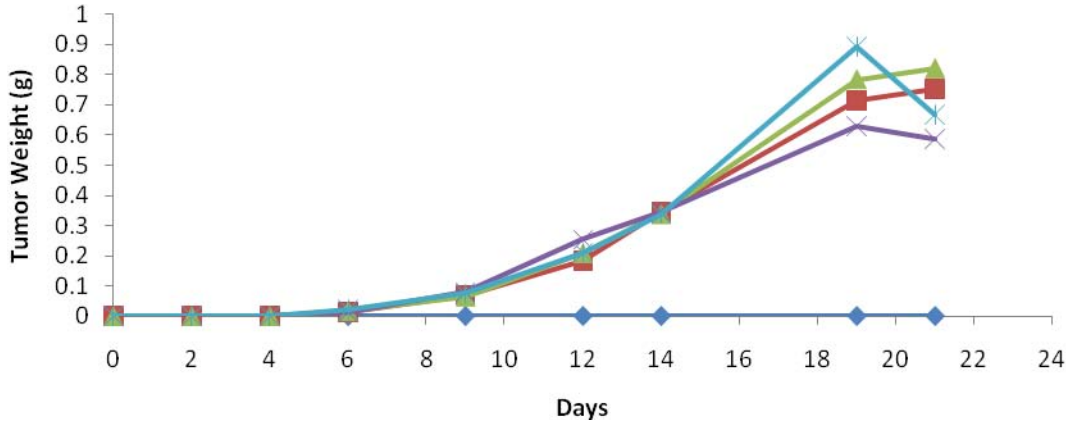


Figure 1. Effect of intramuscular administration of AAV8 carrying MCK-I□ -I□ CMV-cFLIP on body and tumor weight of MCA-26 tumor-bearing in comparison with non-tumor-bearing control (NTB)

CD2F1 mice were intramusculary injected with AAV8 vector carrying MCK-I□ -I□ CMV-cFLIP on tibialis anterior and quadriceps muscle ($3.4x10^{10}$ v.p./muscle) and one week after the mice received MCA-26 tumor inoculation (10^6 cells/mouse) (day 0). (A) Body weight change of mice bearing MCA-26 tumor with or without AAV8 treatment as compared to NTB. (B) Tumor growth rate in MCA-26 tumor-bearing mice with or without AAV8 treatment. The number of mice in each group n=5.

Figure 2

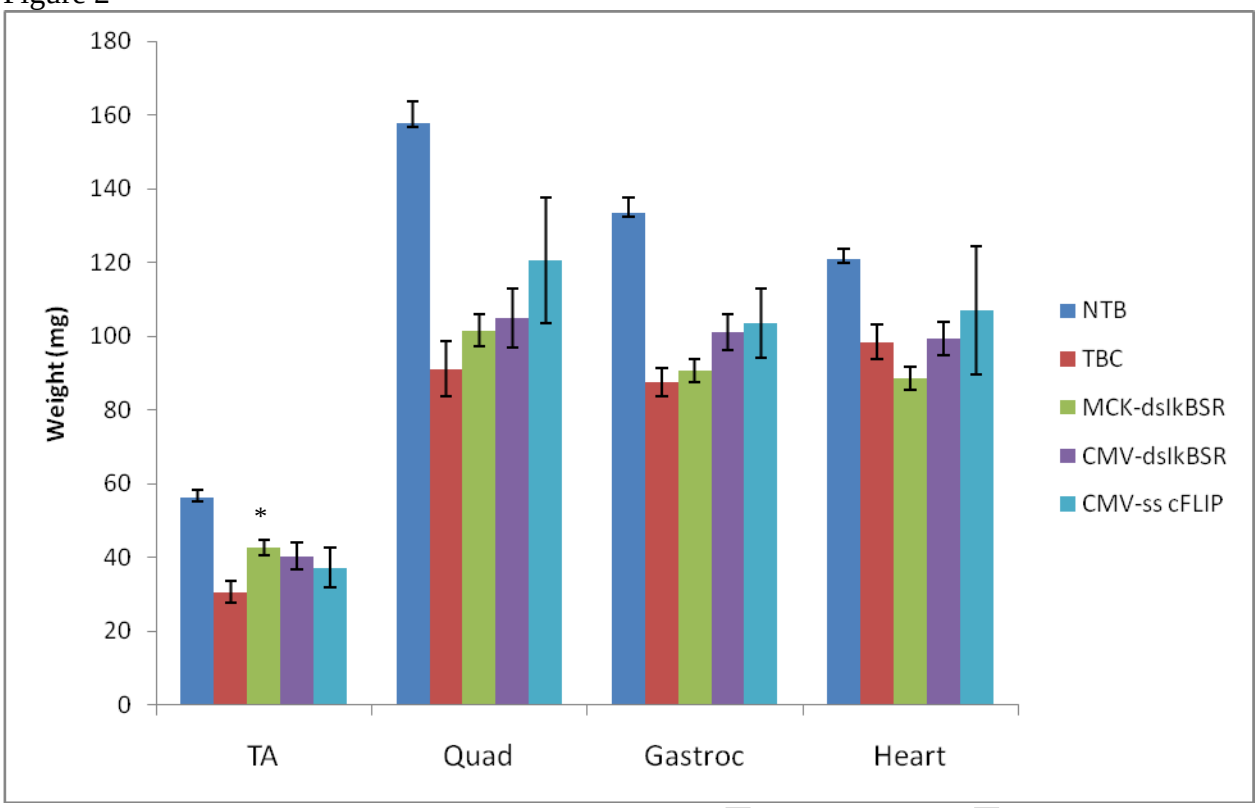


Figure 2. Treatment with AAV8 carrying MCK-I□ -I□ -cFLIP results in improved muscle weight in mice bearing MCA-26 tumor

Mice were sacrificed and hind-limb muscles (TA = tibialis anterior, Quad = quadriceps, Gastroc = gastrocnemius) and heart were collected. The average weight of each individual muscle of NTB and MCA-26 tumor-bearing mice with or without AAV8 treatment are shown. Differences from tumor-bearing without AAV8 treatment (TBC = tumor-bearing control) are shown * = $p < 0.05$.

Figure 3A

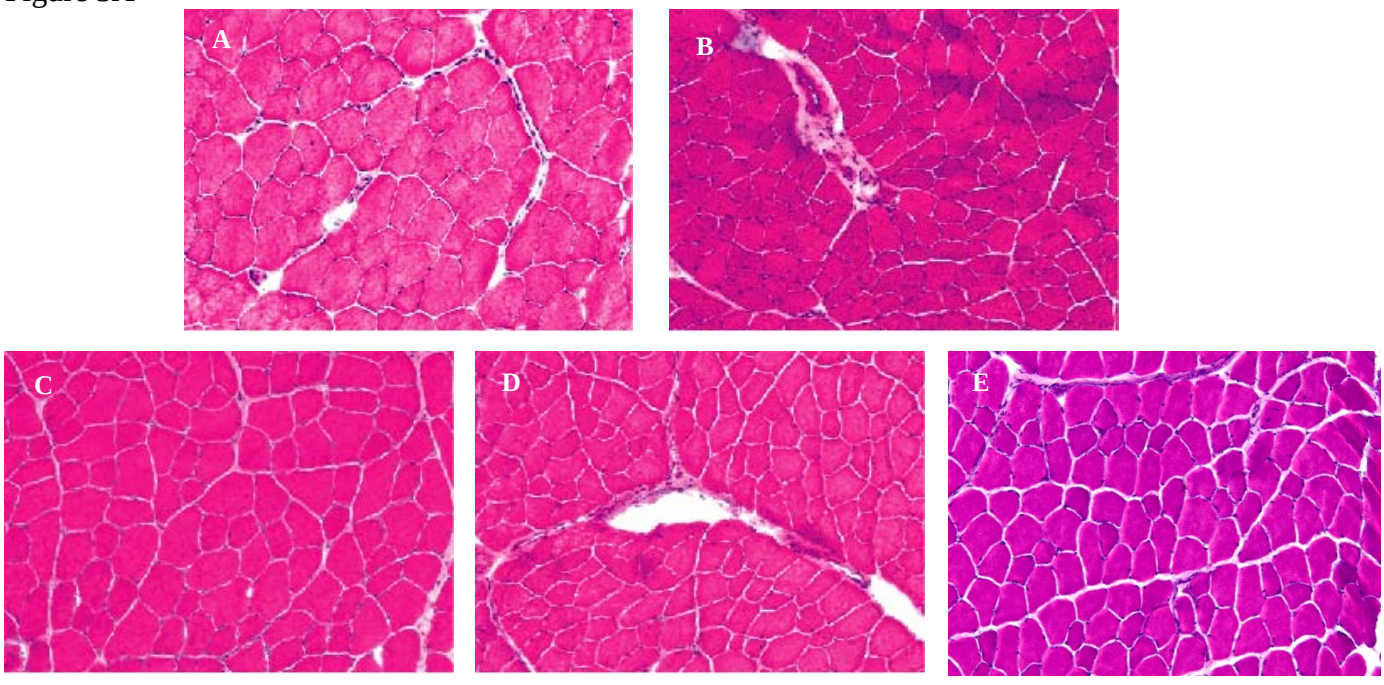


Figure 3B

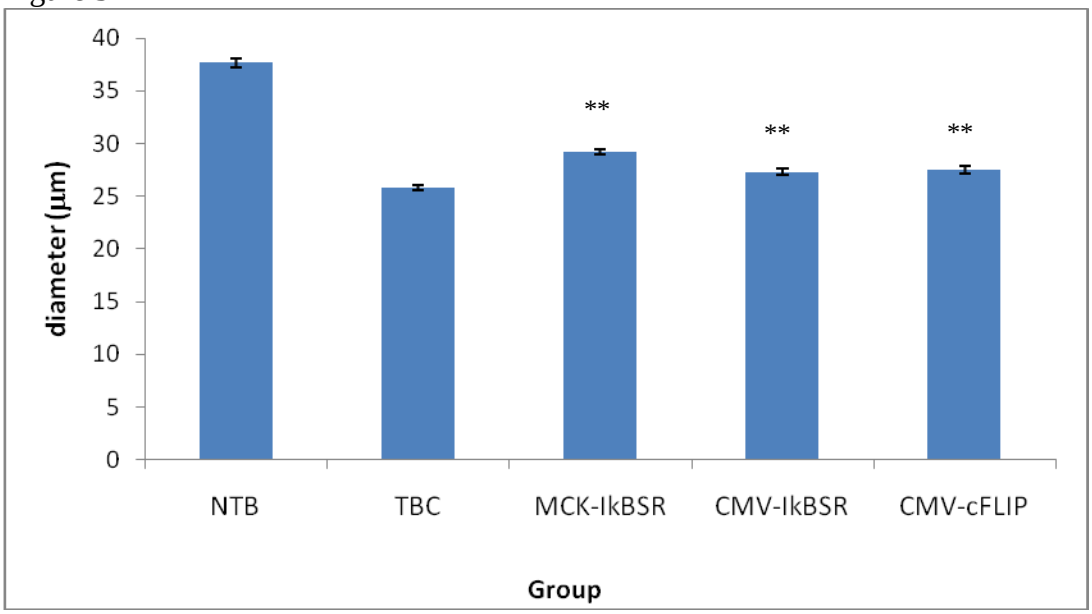


Figure 3. Treatment with AAV8 carrying MCK-IκBSR or CMV-IκBSR or CMV-cFLIP results in increased muscle fiber diameters in mice bearing MCA-26 tumor
Representative H&E staining of TA muscle section is shown in (A); (a) non-tumor-bearing control, (b) MCA-26 tumor-bearing control, (c) AAV8-MCK-IkBSR injected MCA-26 cachexia, (d) AAV8-CMV-IkBSR injected MCA-26 cachexia, (e) AAV8-CMV-cFLIP injected MCA-26 cachexia. The average muscle fiber diameter of each group is shown in (B). Differences from TBC (tumor-bearing control) are shown ** = $p < 0.01$.

Key Research Accomplishments:

- Development of an *in vitro* assay of muscle wasting induced by cancer cytokines
- Demonstration of importance of NF-κB activation as a molecular mechanism for cancer-induced muscle wasting in the *in vitro* assay
- Use of the *in vitro* assay to identify cFLIP as a novel potential therapeutic agent for the treatment of cancer-induced muscle wasting
- Generation of single strand and double strand AAV8 vectors
    - Single strand AAV8 vectors
        - CMV-GFP
        - CMV-c-FLIP
    - Double strand AAV8 vectors
        - MCK-GFP
        - CMV-IκBSR
        - MCK-IκBSR
    - Cloned as plasmid
        - AAV-MCK-D(+)-c-FLIP
- Refinement of 2 cancer cachexia models in mice
- Testing of AAV8 vectors carrying IκBSR and cFLIP for amelioration of cancer cachexia in an *in vivo* murine model

Reportable Outcomes:
Jiang Z, Clemens PR. Cellular caspase-8-like inhibitory protein (cFLIP) prevents inhibition of muscle cell differentiation induced by cancer cells. FASEB J 2006 Dec;20:E1979-E1989.

Sae-Chew P. Clemens PR. A Novel Muscle-Wasting Mouse Model Induced By Human Prostate Cancer Cells: Comparison with a Well Established Model Induced By Murine Colon Adenocarcinoma Cells. Prepared for submission (attached).

Conclusions:
Our studies to date identify an *in vitro* assay that allows us to test molecular therapies that have the potential to treat muscle wasting induced by cancer. We anticipate that these results can be generalized to the treatment of other genetic and acquired causes of muscle wasting. We produced multiple AAV8 vectors with expression cassettes designed to inhibit activation of NF-κB and ameliorate cancer cachexia. We applied several newly developed AAV8 vectors for treatment of cancer cachexia in a murine model.

Vector Core Progress Report
(Bing Wang)

Progress made to date (3-1-06 to 2-28-10):

Vector Core of the DOD project (W81XWH-06-1-0406)
Sub-title: The structure, production and purification of adeno-associated viral, adenoviral and retroviral vectors"

Introduction:

The Molecular Therapy Laboratory (MTL) in the Department of Orthopaedic Surgery is focusing on viral based gene transfer and stem cell technologies for musculoskeletal diseases, mainly by using recombinant adeno-associated viral (rAAV), retro-viral and lenti-viral vectors which represent the most promising approaches to aid in the repair and regeneration of muscle, bone, ligament, tendon, and joint capsules. In the meanwhile, MTL also serves as a Vector Core of a DOD project (W81XWH-06-1-0406, PI: Dr. Johnny Huard) for the design, construction and production of a lots of viral vectors which are used to gene transfer and genetic engineering of stem cells in the whole project.

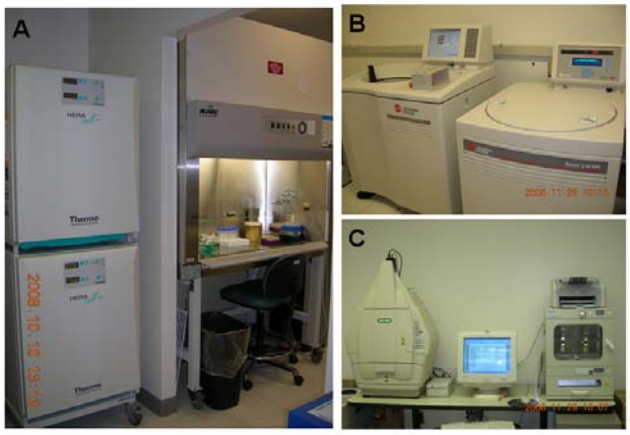


Figure 1. Major Equipment

The Vector Core is located in the Biomedical Science Tower, room E1603. Total dedicated Vector Core space is approximately 500 square feet for molecular biology and viral vector production. Inventory of major equipment includes one tissue culture hood and one temperature-controlled CO2 incubator for cell culture and viral vectors production (**Figure 1 A**). In addition, Vector Core is also equipped with one Beckman TM L-100 XP Ultracentrifuge, one Beckman Avanti TM J-20 XP high-speed centrifuge for the purification of plasmid DNA and viral vectors (**Figure 1B**). Moreover, one Beckman refrigerated table-top centrifuge, two microfuges, one refrigerator, one BioRad PCR machine, one UV spectrophotometer, and one BioRad gel documentation camera (**Figure 1C**), and two BioRad electric gel transfer apparatuses will be used for molecular analysis and vectors construction. Also, one Nikon inverted fluorescent microscope and -30ºC and -80ºC freezers, and one liquid nitrogen tank are established for evaluating the expression of report genes, storages of plasmid DNA, viruses and cell clones.

Body

Development and production of AAV vectors

The Vector Core is actively engaged in multiple applications of rAAV vectors for gene therapy, including different serotypes of AAV vectors, both single and self-complementary AAV vectors, inducible vector system. Moreover, we developed the different promoters in AAV vectors and gene-silencing AAV vectors. As a vector core, we collaborated with many laboratories internal and external the University of Pittsburgh. Our goal is to develop very useful viral vectors for directly gene transfer and to aid in the characterization of the cells, track the cells and genetically modify the cells to express beneficial proteins to enhance their transplantation efficiency. Such as Decorin, mini-dystrophin, and myostatin propeptide genes etc. were constructed in AAV vectors. As described in following, we produced lots of AAV vector for the aims in this project:

1. AAV2-D(+)-CMV-eGFP

2. AAV6-D(+)-CMV-eGFP
3. AAV8-D(+)-CMV-eGFP
4. AAV9-D(+)-CMV-eGFP
5. AAV8-D(+)-CMV-eGFP
6. AAV8-D(+)-tMCK-eGFP
7. AAV2-D(+)-CB-eGFP
8. AAV2-1060-hrGFP
9. AAV2-1070-hrGFP
10. AAV2-1060-Gluciferase
11. AAV2-1070-GLuciferase
12. AAV2-CMV-sflt1
13. AAV2-D(+)-BMP2-spA
14. AAV2-D(+)-BMP2-SV40
15. AAV2-D(+)-CMV-TGFβ1
16. AAV2-CMV-minidystrophin (3858)
17. AAV2-dMCK-minidystrophin (3858)
18. AAV6-D(+)-CMV-BMP4
19. AAV2-D(+)-CMV-VEGF
20. AAV1-CMV-Decorin

Development and production of lenti-viral vectors

In the meanwhile, we also updated the BSL-2 to BSL-2+ for development of lenti-viral vectors using in the aims of this project, including green (GFP) and red (RFPN) reporter genes and silencing lenti-viral vectors targeting to human and mouse VEGF genes.

1. lenti-CMV-GFP
2. lenti-CMV-RFPN
3. lenti-CMV-Luciferase
4. lenti-minidytrophin (3849)-GFP
5. lenti-shRNA-mouse VEGF-GFP
6. lenti-shRNA-human VEGF-GFP

Efficiency of viral vectors

To test the efficiencies of AAV and lenti-viral vectors, we made different viral vectors and the sequential dosages of reporter viral vectors to test which viral vectors could achieve the higher transduction efficiency and lower dose vector loading, such as green fluorescent protein (GFP) and red fluorescent protein in nucleolus (RFP-N), as shown in **Figure 3**. Also, we used same strategy to optimize the serotype of AAV vector that appears a variety of AAV transduction in the different cells and tissues. The advantage of the GFP or RFP reporter is that it can be directly visualized on the whole limb as well as on the cryo-sections. Its fluorescent intensity could also be quantitated by the Nikon microscope imaging software we have been using in the lab. Usually, at 3 weeks following implantation, at which time the GFP gene expression is expected to reach a considerable level for detection and quantitation, the animals would be sacrificed and skeletal muscles, hearts and liver will be collected for photographs for GFP or RFP expression and for microscopic photographs and quantitations from the intensity of the fluorescence after cryo-thin-section.

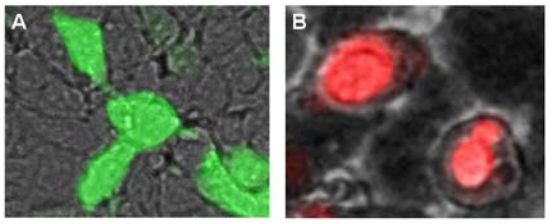


Figure 3. Transduction of AAV- and lenti-viral vectors in 293 cultured cells 36 hours post-infection. (A) shows AAV-GFP, and (B) represents Lenti-RFP-N

To achieve *ex vivo* gene transfer into *mdx* skeletal muscle using lenti-minidystrophin gene, we constructed a fusion protein containing minidystrophin and GFP reporter gene. GFP reporter can monitor the proliferation and differentiation of muscle derived stem cells *in vivo*, and it can also represent the expression of mini-dystrophin gene directly under microscopy, without toxicity and immune response. In preprimary study, we created this fusion protein construct in a pEGFP-N1 plasmid, and then cloned it into lenti-viral vector under the control of the CMV promoter. As shown in **Figure 4**, the fusion protein was expressed in both C2C12 and 293 cells after transfection.

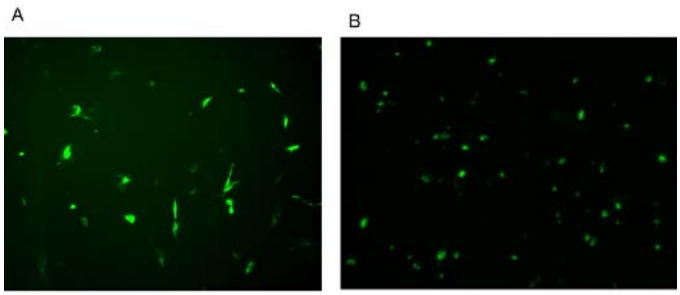


Figure 4. The expression of mini-dystrophin-GFP fusion protein after transfection. (A) 24 hours post-transfection of Mini-dystrophin-GFP fusion in C2C12 by Biotex. (B) 4 days post-transfection of Lenti-Mini-dystrophin-GFP fusion in 293 cells,

Achievements

Invited review book "Regenerative therapy for the musculoskeletal system using recombinant adeno-associated viral vectors"

1. **Bing Wang** and Johnny Huard, 2008, the Chapter "Gene therapy for the treatment of muscle disorders"
2. **Bing Wang** and Freddie H. Fu, 2008, the Chapter "rAAV vectors for soft tissue reconstruction"

Publication and abstracts in 2008 and 2009

1. Chunping Qiao et al. *Human Gen Therapy, 2008, 19 (3), 241-253.*
2. Bing Wang et al. *Gene Therapy, 2008, 15 (15), 1099-1106.*
3. Bing Wang et al. *Gene Therapy, 2008, 15 (22), 1489-1499, correspondence author.*
4. Juan Li etal. *Hum Gene Ther. 2008, 19:958-964.*
5. Bing Wang et al. *Journal of Orthopaedic Research, 2009,27:421-426, correspondence author.*
6. Bin Li et al. *Journal of Biomechanics, 2008, 41:3349-3353.*
7. Michael Y. Mi et al. *Molecular Therapy, 2008, vol.16, suppl 1, S103, correspondence author.*
8. Peiqi Hu et al. *Molecular Therapy, 2008, vol.16, suppl 1, S201*
9. Bhanu M et al. *Molecular Therapy, 2008, vol.16, suppl 1, S293*
10. Joseph Kornegay et al. *Molecular Therapy, 2008, vol.16, suppl 1, S374*
11. Ying Tang; Bin Li, James H-C Wang, Johnny Huard, Melessa Salay, Bing Wang. *In vitro* AAV-mediated gene transfer in human fibroblasts. *54th Annual Meeting of Orthopaedic Research Society, March 1st – March 6th, 2008*
12. Bin Li, Micheal Lin, Ying Tang, Bing Wang, James H-C Wang. Micropatterned C2C12 cells exhibit enhanced differentiation into myotubes – a novel study using cell traction force microscopy. *54th Annual Meeting of Orthopaedic Research Society, March 1st – March 6th, 2008*
13. Guangheng Li, Bo Zheng, Andres J. Quintero, Arvydas Usas, Bing Wang, Laura B. Meszaros, Karin A. Corsi, Johnny Huard. Therapeutic Utility of Heterotopic Ossification Induced by AAV-BMP4 in Skeletal Muscle. *54th Annual Meeting of Orthopaedic Research Society, March 1st – March 6th, 2008*
14. Mi, Y.M; Tang, Y; Li, G-H; Salay, M.N; Niyibizi, C; Huard, J; Wang, B. AAV Based *Ex Vivo* Gene Therapy in Rabbit Adipose Stem/Progenitor Cells for Osteogenesis. *55th Annual Meeting of Orthopaedic Research Society, February 22 – 25, 2009, Las Vegas, Nevada.*
15. Tang, Y; Reay, D.P; Salay, M.N; Mi, Y.M; Clemens, P.R; Guttridge, D.C; Robbins, P.D.; Huard, J; Wang, B. Delivery of Dominant Negative IKKs by Recombinant Adeno-associated Virus: Efficient Improvement in Muscle Regeneration in *mdx* Mice. *55th Annual Meeting of Orthopaedic Research Society, February 22 – 25, 2009, Las Vegas, Nevada.*

16. Michael Y. Mi, Ying Tang, Melisa N. Salay, Guangheng Li, Johnny Huard, Freddie H. Fu, Christopher Niyibizi and Bing Wang. *AAV ex vivo Gene Therapy for Osteogenesis and Chondrogenesis. Submitted to 12th Annual meeting of ASGT (May 27-30, 2009, San Diego, California).*
17. Daibang Nie, Dong Wei, Michael Y. Mi, Ying Tang, Allan Z. Zhao, Yifan Dai, Johnny Huard, and Bing Wang. Muscle Specific Gene Transfer of the PEPCK-C Improves Physical Activity in Mice. *Submitted to 12th Annual meeting of ASGT (May 27-30, 2009, San Diego, California).*
18. Ying Tang, Daniel P. Reay, Melessa N. Salay, Michael Y. Mi, Paula R. Clemens, Denis C. Guttridge, Paul D. Robbins, Johnny Huard, Bing Wang. AAV Based Blocking of NF-kappa B Pathway Improves Muscle Regeneration in *mdx* Mice. *Submitted to 12th Annual meeting of ASGT (May 27-30, 2009, San Diego, California).*

Awards

1. Ying Tang et al. *In vitro* AAV-mediated gene transfer in human fibroblasts. Oral presentation given at the 54th Annual Meeting of Orthopaedic Research Society, March 1st – March 6th, 2008.
2. Michael Y. Mi et al. AAV Based *Ex Vivo* Gene Therapy in Rabbit Adipose Stem/Progenitor Cells for Osteogenesis" was accepted as a podium presentation at the 55th Annual Meeting of Orthopaedic Research Society, February 22 – 25, 2009, Las Vegas, Nevada.

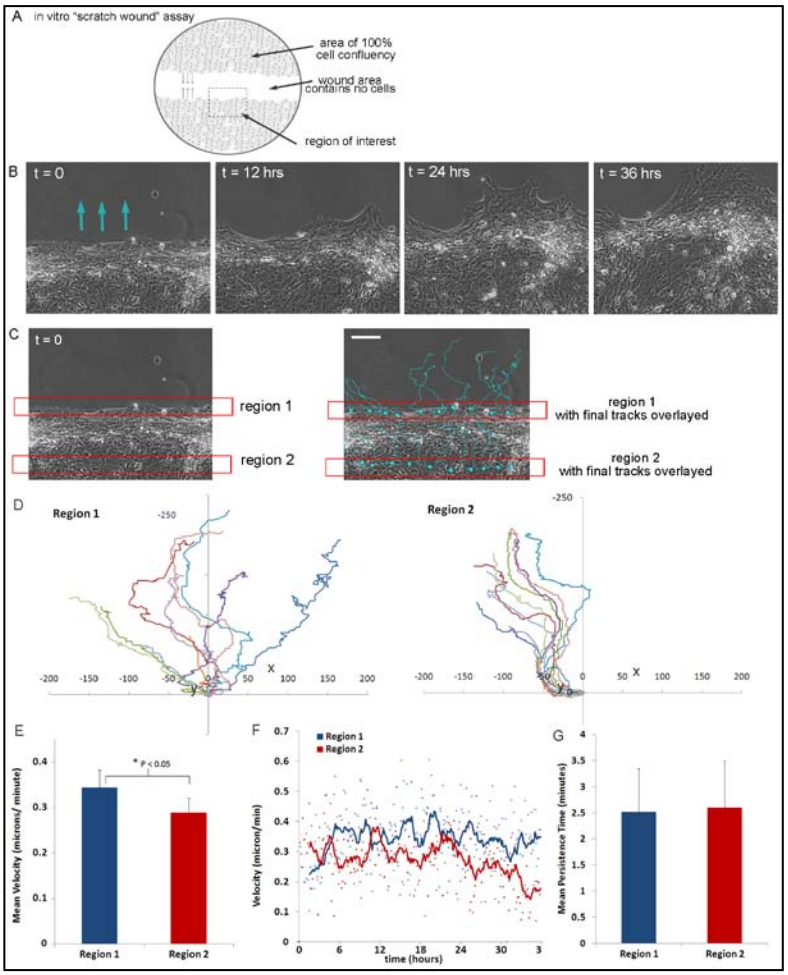
Conclusion

The Vector Core for this project provided a series of viral vectors containing reporter and therapeutic genes proposed in aims. Also, it will develop the Tet-On retro-viral vector system to express a VEGF-LacZ fusion protein.

Bioreactor Core Progress Report
(Johnny Huard and Bridget Deasy)

Progress made to date (3-1-06 to 2-28-10):

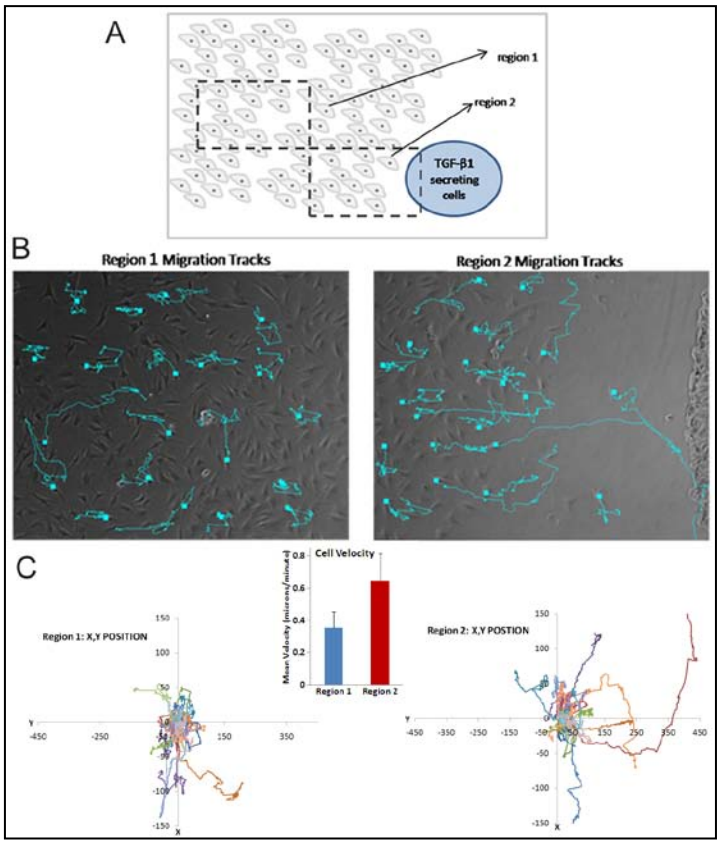
In 2009, the Bioreactor Core expanded to include a 3rd imaging system. The addition of another system has allowed us to develop existing projects more in depth, to add new collaborators, and to dedicate some resources to additional research and development of the technology. In the past year, we have developed new approaches and projects which utilize time-lapsed microscopy. Two examples of our new developments are assays for 1) cell migration and 2) cell chemoattraction. Below we describe these assays by providing a brief example of their use. In addition to the example use, the same assays could be used by other collaborators within a variety of contexts.



In the first example shown in Figure 1, we illustrate an approach to the use of time-lapsed imaging to examine cell migration. **Figure 1.** **(A)** In the "scratch wound" assay, a confluent layer of cells is wounded by manually scratching a region of the plate to remove all cells. **(B)** Cell migration into the "wound" occurs over several days. Variables in these experiments may include different cell types, different supplements to the growth media or alterations in the binding proteins on the cell surface. In this example, the total wound area was ~800-micron-wide (entire wound not shown) and we observed cells on the edge of the wound to infiltrate about 1/3 of the scratch wound area by 36 hours. **(C)** We quantified the movement of cells in 2 different regions - cells in Region 1 were at the edge of the scratch wound, while cells in Region 2 were ~200 microns away from the scratch edge. **(D)** Cell tracks were obtained from the x-y positions of each cell for each 10-minute interval frame over the course of 36 hours (216 image frames per cell). Cells in both regions moved in the direction towards the scratch wound, with no cells migrating inwards toward the confluent region. **(E)** The mean cell velocity for cells at the free edge had a significantly larger mean cell velocity (0.34 ± 0.04 microns/min) as compared to cells within the confluent Region 2 away from the edge (0.29 ± 0.03 microns/min, p = 0.046). **(F)** There was no change in cell velocity for cells at the scratch wound edge during the course of the 36 hours, however, there was a significant decrease in cell velocity over time for cells in the confluent Region 2 (p = 0.036). **(G)** There was no difference in the mean directional persistence of cell migration for the 2 regions (Region 1; 2.5 ± 0.8 minutes, and Region 2; 2.6 ± 0.9 minutes).

In the second example, we have used, time-lapsed imaging to examine cell chemoattraction towards a growth factor, GF, involved in many aspects of cell behavior including cell proliferation and differentiation. In

Figure 2, cells which expressed the GF were cultured and pelleted. Non- transduced cells and the transduced pellet were plated according to Figure 1A. Cell tracks are displayed over the visible image frame 1 of the image sequence (Figure 2B). Bone marrow cells in Region 1 appear to display random migration tracks (Figure 2B,C). Some cells in Region 2 appear to exhibit non-random motion in the direction of the chemoattracting pellet (Figure 2B,C). Cells in Region 1 show movement in all directions, whereas cells from Region 2 show tracks that move in the positive x-direction, towards the pellet, and no tracks are observed to move in the direction opposite to the pellet (or – x direction, Figure 2 C). In addition, cell velocity (microns /min) was significantly higher for cells nearer to the GF signal as compared to cells farther away from the signal.



Here we show the use of time-lapsed imaging to follow cell movement, and we assess whether the direction is random or directed. The bone marrow cells in the region closer to the GF – secreting pellet show cell movement in the direction of the pellet with higher cell velocity, while bone marrow cells farther away from the pellet showed more random tracks. Additional studies will be performed to examine the spatio-temporal range of the GF signal in this assay. The doses of the GF signal will also be examined to determine if cells at greater distances can be recruited to the site of the GF signal. These studies have implications for chemoattraction of bone marrow cells to sites of cartilage injury or repair sites initiated by microfracture surgeries.

Live cell imaging provides a tool to capture unique data of cell behavior. The technological advancements in computer processing, digital imaging, robotic controls and data handling, create a robust tool for basic and applied biomedical research. Many different types of questions may be addressed using these systems. Here, we include examples of analysis of cell migration and chemoattraction. This technology greatly enhances cell science by providing a unique dataset to quantify numerous temporal changes in cell populations. Practically, the technology also drastically reduces man-hours, consumes fewer laboratory resources, and provides standardization to assays for cell research.

Key Core Users and Collaborative Projects (a sample of several core collaborations)

1. Dr. Bridget Deasy, Department of Orthopedic Surgery, University of Pittsburgh Medical Center
2. Dr. Johnny Huard, Department of Orthopedic Surgery, University of Pittsburgh Medical Center
3. Dr. Yong Li, Department of Orthopedic Surgery, University of Pittsburgh Medical Center
4. Dr. Fabrisia Ambrosio Department of Rehabilitation Sciences, University of Pittsburgh Medical Center
5. Dr. Partha Roy. Dept of Bioengineering, University of Pittsburgh
6. Dr. Alan Wells, Departments of Pathology, University of Pittsburgh
7. Dr. Steven Badylak, Department of Surgery, University of Pittsburgh Medical Center
8. Dr. Ipsa Banjere, Department of Chemical Engineering, University of Pittsburgh

2009-2010 JOURNAL and ABSTRACT PUBLICATIONS which include Bioreactor Core Technology

JOURNAL PUBLICATIONS

1. RC Schugar, KE Wescoe, SM Chirieleison, JJ Nance, Y Askew, BT Schmidt, JM Evron, B Peault and BM Deasy. 2009. In situ Identification of CD146 Stem Cells in Primitive Human Umbilical Cord Stroma with High Isolation Yield, High Expansion and Phenotype Stability for Regenerative Therapies. *Journal of Biomedicine and Biotechnology.* Volume 2009, Article ID 789526, doi:10.1155/2009/789526.
2. Deasy, BM*, Chirieleison SM, Witt, AM, Peyton, MJ, Bissell, TA. 2009. Tracking stem cell function with computers via live cell imaging: Identifying donor variability in human stem cells. *Operative Techniques in Orthopedics*. *In press. * corresponding author.*
3. Chirieleison, SM, Scelfo, CC, Bissell, TA, Anderson, JE, Koebler, D, Yong Li, Deasy, BM. Robotic imaging systems for biological study of dynamic cell behavior. *Under review.*
4. Chirieleison SM , Feduska JM, Schugar RC, Sanford SL, Scelfo CC, Askew Y, Deasy BM. 2010. Human Muscle-Derived Cells Isolated by Differential Adhesion Rates Exhibit Differences in Phenotype and Contribution to Skeletal Muscle Regeneration in mdx/SCID model. *In submission.*
5. L Leloup, H Shao, Yong Ho Bae, B Deasy, D Stolz, P Roy, and A Wells. The Protease M-Calpain (Calpain 2) Is Regulated By Its Localization At The Plasma Membrane, Notably During Wound Healing Process. 2010. *In review.*

ABSTRACT PUBLICATIONS (selected list)

1. Deasy, BM, Chirieleison SM, Witt, AM, Bissell, TA, Peyton, M J. 2010. Tracking stem cell activity with computers via live cell imaging: Identifying donor variability in human umbilical cord stem cells. *Pittsburgh Orthopedic Journal*
2. Bissell, TA, Chirieleison SM, Witt, M, Kline, DE; Deasy, B M. 2010. Identification of Potent Human Muscle Stem Cells by Live Cell Imaging. *Pittsburgh Orthopedic Journal.*
3. Anderson JE, Chirieleison, SM, Scelfo CS, TA. Bissell, Yong Li, Koebler DJ, Deasy BM. 2010. Robotic imaging systems for biological study of dynamic cell behavior in cell migration assays. *Pittsburgh Orthopedic Journal*
4. Anderson JE, Yao V, Chu CR, Deasy B. 2010. The Use Of Time-Lapsed Microscopy To Study Cell Migration And Chemoattraction. *Pittsburgh Orthopedic Journal*
5. Witt, AM, Nance, JJ; James, SD;Wescoe, KE; Usas, A; Deasy, B M. 2010. Human Donor Variability in Osteogenesis of Human Umbilical Cord Stem Cells. *Pittsburgh Orthopedic Journal*
6. Nance, J J; Wescoe, K E; Witt, A M; Deasy, B M Induction of Chondrogenesis in Umbilical Cord Derived Stem Cells in Hypoxic Environment. 2010 Orthopaedic Research Society; New Orleans, LA
7. Bissell, T A; Chirielsion S C; Witt, M; Kilne, D; Deasy, B M. Potent Human Muscle Stem Cells are Identified by Live Cell Imaging. 2010 Orthopaedic Research Society; New Orleans, LA
8. James, SD; Witt, AM; Nance, JJ; Chirieleison, SM; Bissell, TA; Usas, A; Piganelli , JD; Deasy, BM. 2009. Effect of cell sex on the osteogenic differentiation of stem cells of human umbilical cords. Pittsburgh Tissue Engineering Initiative Summer Research Program.
9. Witt MCS, Bissell T, Kline D, Witt AM, Nance JJ, Chirieleison SM, Deasy BM. 2009. The Use of Software in Order to Find Qualities of Stem Cells. University of Pittsburgh School of Engineering, Investing Now Summer Program. POSTER
10. Scelfo CC, Chirieleison SM, Witt AM, Bissell TA, Deasy BM. 2009. Robotic Imaging Systems for Biological Study of Dynamic Cell Behavior. *Biomedical Engineering Society Annual Meeting*; Pittsburgh, PA, USA. *POSTER*

11. Chirieleison SM, Nance JJ, Witt AM, Schugar RC, Schmidt BT, Deasy BM. 2009. Viability of Human Umbilical Cord as Alternative Cell Source for Muscle-Directed Therapy. *Pittsburgh Orthopaedic Journal 2009;* Pittsburgh, PA, USA.
12. Chirieleison SM, Witt AM, Flythe SY, Deasy BM. 2009 Live Automated Cell Imaging, Examination of Human Stem Cell Population Dynamics Under Growth Factor Treatment. *Pittsburgh Orthopaedic Journal 2009;* Pittsburgh, PA, USA.
13. Chirieleison SM, Nance JJ, Witt AM, Schugar RC, Schmidt BT, Deasy BM. 2009. Examination of Human Umbilcial Cord MSCs for Muscle-Directed Therapy. *International Society for Stem Cell Research;* Barcelona, Spain. *POSTER*
14. Chirieleison SM, Witt AM, Flythe S, Deasy BM. 2009. Live Automated Cell Imaging, Examination of Human Stem Cell Population Dynamics Under Growth Factor Treatment. *Midwest Tissue Engineering Consortium;* Pittsburgh, PA, USA. *PODIUM*
15. Wescoe KE, Bencherif SA, Sun S, Schugar RC, Washburn NR, Deasy BM. 2009. The Phenotype and Viability of Novel, Photoencapsulated Human Umbilical Cord (hUC) Stem Cells Pre- and Post- Mechanical Stimulation. ISSCR. Barcelona, Spain. *POSTER*
16. Wescoe KE, Schugar RC, Askew YS, Deasy BM. 2009. The Effects of Ex Vivo Expansion on the Chondrogenic Activity of Novel Umbilical Cord Stem Cells. MidWest Tissue Engineering Consortium. Pittsburgh, PA, USA. *PODIUM*
17. Chirieleison SM, Schugar RC, Schmidt BT, Deasy BM. 2009. Viability of Human Umbilical Cord as Alternative Cell Source for Muscle-Directed Therapy. Orthopaedic Research Society Annual Meeting; Las Vegas, NV, USA. *PODIUM / POSTER*

Micro-CT Core Progress Report
(Johnny Huard and Arvydas Usas)

Progress made to date (3-1-06 to 2-28-10):

We continue to use the vivaCT 40 (Scanco Medical) imaging system for nondestructive 2-D and 3-D visualization and quantitative analysis of mineralized matrix volume, density and other structural parameters of bone tissue. VivaCT 40 system enables us to perform live animal imaging on the same animal at different time points; therefore we are able to reduce the number of experimental animals and cut the cost for animal housing and care. We are also performing CT imaging of various animal body parts and tissues (calvaria, spine, extremities, muscles, etc.) harvested after animal euthanasia and stored in fixative solution for extended period of time.

We are using continuous cell pellet culture imaging to detect matrix mineralization after osteogenic stimulation in vitro. This system we believe can be very useful for the screening of cellular candidates for bone tissue engineering.

We continue to investigate skeletal muscle angiogenesis using contrast-enhanced micro-CT imaging. One method that has been applied involves transcardiac perfusion and injection of Microfil® silicone rubber compound that polymerize after 4-6 hours in room temperature and blood vessel network in post-mortem tissues becomes radiopaque and easily detectable by micro-CT. Another contrast agent for vascular imaging is Fenestra VC, which can be administered repetitively to animals via single dose administration into tail vein. With Fenestra VC, the delayed uptake by liver cells produces an agent with superior blood pool imaging properties that last for several hours after injection. We performed several pilot experiments to investigate whether Fenestra VC can be used to visualize peripheral vasculature in living mice. We are collaborating with researchers from other institutions to develop scanning protocol for the digital subtraction angiography which necessitates obtaining CT scans prior to contrast injection and after without repositioning of the scanning object.

During the report period the use of the micro-CT contributed to the following research accomplishments by the members of our laboratory:

1. Conversion of transformed stem cells to osteogenic cells using siRNA. Barry D, et al. Podium presentation at the ORS meeting 2010, New Orleans, LA.
2. The use of ACL derived blood vessel progenitors for the ACL reconstruction. Poster at the ORS meeting 2010, New Orleans, LA.
3. Treatment using a therapeutic peptide inhibitor against the stress mediator NF-kB delayed age-dependent spinal degeneration in an accelerated aging mouse model. Vo N, et al. Podium presentation at the NASS meeting 2009, San Francisco, CA.
4. Spatial regulation of bone formation in vivo. Cooper GM, et al. Podium presentation at the ACPCA annual meeting 2009, Scottsdale, AZ.
5. The dose of growth factors influences the synergistic effect of vascular endothelial growth factor on bone morphogenetic protein 4-induced ectopic bone formation. Li G, et al.Tissue Eng Part A. 2009 Aug;15(8):2123-33.
6. Inkjet-Based Biopatterning of Bone Morphogenetic Protein-2 to Spatially Control Calvarial Bone Formation. Cooper GM, et al. Tissue Eng Part A. 2010 Feb 16. [Epub ahead of print]
7. Accelerated aging of intervertebral discs in a mouse model of progeria. Vo N, et al. (Manuscript accepted for publication in JOR).
8. Role of PI3K, ERK1/2, and p38 MAPK pathways in BMP4-induced matrix mineralization by MDSCs. Payne-Corsi K, et al. (Manuscript in revision in Tissue Eng).
9. Inhibition of NF-kB delays the onset of age-related degenerative diseases. Tilstra JS, et al. (Manuscript submitted).

10. The increased presence of multipotent PCs and EPCs in the peripheral region of fetal and adult menisci. Osawa A, et al. (Manuscript submitted to JBJS).
11. Myoendothelial cells: a novel stem cell population from adult human skeletal muscle. Zheng B, et al. (Manuscript in preparation).
12. Identification and characterization of chondrogenic progenitor cells in adult skeletal muscle. Li GH, et al. (Manuscript in preparation).
13. Human skeletal muscle cells can undergo matrix mineralization in vitro and facilitate bone repair after delivery on SIS scaffold. Payne-Corsi K., Usas A, et al. (Manuscript in preparation).

Following is the list of current research projects that involve the use of micro-CT core:

1. Guided bone regeneration and bone defect healing using spatial BMP4 protein distribution on prefabricated fibrin and collagen scaffolds using 3D printing technology. (In collaboration with Dr. G. Cooper).
2. Osteogenic potential of human muscle-derived cells seeded on SIS scaffolds after osteogenic stimulation in vitro and transplantation into critical-size calvarial defect (In collaboration with Cook MyoSite, Inc.).
3. Osteogenic potential of different populations of human muscle-derived cells (unsorted, myo-endo, pericytes) transduced with BMP4, or based on high and low ALP expression after FACS. (NIH funded project).
4. Evaluation of skeletal muscle angiogenesis after laceration, contusion or strain injury, electrical stimulation and physical exercise in mice, and its relationship to development of fibrosis (In collaboration with Dr. F. Ambrosio).
5. Evaluation of skeletal muscle vasculature and vertebral bone structure in mouse model of accelerated aging (In collaboration with Dr. L. Niedernhofer and Dr. N. Vo).

Administrative Core Progress Report
(Johnny Huard, James Cummins and Matthew Bosco)

INTRODUCTION:

The Administrative Core of the Stem Cell Research Center (SCRC) is directly responsible for ensuring the proper function and integration of the Research Laboratories (comprising the Core Research Laboratories, Affiliated Laboratories, and Research Core Facilities), the Clinical Trials Unit, and the Educational Programs that constitute the SCRC. The Administrative Core provides administrative services to all SCRC personnel, supports the ongoing activities of the SCRC, and provides a mechanism for regular evaluation of the SCRC. The Administrative Core also is responsible for fulfilling the secretarial, budgetary, and grant application and manuscript preparation needs of SCRC personnel. In addition, this Core also facilitates collaboration between SCRC researchers and scientists working in designated Collaborative Institutes or other, non-affiliated laboratories.

BODY:

The Administrative Core continues to provided managerial and financial oversight for the entire Department of Defense Program. The center administrator, Matthew Bosco, and Senior Scientist, James Cummins have played key roles in facilitating and administering all of the various projects within the Program. Mr. Bosco and Mr. Cummins help ensure that each project functions smoothly and efficiently while maintaining continuous communication between the various investigators and institutions.

The Administrative Core holds weekly/biweekly seminar series for SCRC researchers, affiliates, collaborators, and other interested scientists. The goal of each of these events was to bring in highly regarded scientists who are performing cutting edge research in cellular therapeutics. In addition to serving as a forum in which SCRC scientists can interact with other researchers who share similar interests, this series has helped to introduce the invited speakers to the SCRC and has also helped to promote the development of ongoing collaborations with these well-regarded research scientists. The goal of these meetings is to help SCRC researchers hone their scientific critical analysis skills and, by so doing, improve their own research and writing abilities.

The use of suramin to improve skeletal muscle healing after contusion injury

*Nozaki M, *Li Y, *Zhu J, *Ambrosio F, **Fu FH, +*Huard J
*Stem Cell Research Center, Children's Hospital of Pittsburgh and Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA
jhuard@pitt.edu

INTRODUCTION:

Muscle injuries are very common musculoskeletal problems encountered in sports medicine. Although this type of injury is capable of healing, an incomplete functional recovery often occurs, depending on the severity of the blunt trauma. Complete functional recovery is hindered by the development of scar tissue formation, which typically appears during the second week following muscle injury. We have reported that TGF-β1 is a major factor in triggering the fibrotic cascade within injured skeletal muscle [1]. The use of antifibrosis agents, such as suramin, that inactivates TGF-β1 can reduce muscle fibrosis and consequently improve muscle healing after injury. The ability of suramin to block the fibrotic effect of TGF-β1 and reduce the fibrosis makes this molecule well suited for use in applications to improve muscle healing after injury. We already reported that suramin can effectively prevent muscle fibrosis and enhance muscle regeneration in the lacerated and strain-injured muscle [2,3]; however, it is still not known if suramin can improve muscle healing after contusion injury, which is the most commonly encountered muscle injury. Furthermore it has remained unclear whether this enhanced muscle regeneration is a direct effect of suramin. We performed this study to examine whether suramin would promote differentiation of myogenic cells *in vitro* and improve injured muscle healing by enhancing regeneration and reducing fibrosis *in vivo*, using an animal model of muscle contusion.

MATERIALS AND METHODS:

Muscle-derived stem cell differentiation assay: Muscle-derived stem cells (MDSCs) were isolated from wild type mice(C57BL/6J) via the modified preplate technique [4]. MDSCs (10^4 cells/well) were seeded into 12 well plates and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 10% horse serum, 0.5% chicken embryo extract, and 1% penicillin/streptomycin. After 24 hours, the medium was replaced with differentiation medium (DMEM containing 2% horse serum and 1% penicillin/streptomycin) containing different concentration of suramin (0, 1, 10, 100μl/ml). After another 24 hours, the medium was replaced with only differentiation medium. All cells were grown at 37°C in 5% CO_2. Three days after incubation the fusion index was assessed by counting the number of nuclei in differentiated myotubes as a percentage of the total number of nuclei.

Immunocytochemistry in vitro: Immunocytochemistry was performed on the cells *in vitro* to examine their expression of fast myosin heavy chain (MyHC). Mouse anti-MyHC (1:250; Sigma) was used as the primary antibody, and biotinylated anti-mouse IgG (1:200; Vector) was used as the secondary antibody. Streptavidin 555 conjugate (1:500; Molecular Probes) was applied to detect the secondary antibody.

Animal model: The muscle contusion model was developed in normal wild-type mice (7-10 weeks, average weight 24.0g). A 17g stainless steel ball was dropped through an impactor from a height of 100cm onto the animal's tibialis anterior (TA) muscle. Mice were divided into 4 groups (5mice/group). Different concentrations of suramin (0, 2.5, 5, 10mg in 20μl of Phosphate-buffered solution [PBS]) were injected intramuscularly two weeks after injury. Cryostat sections of muscles (10μm in thickness) were obtained and histologically stained (hematoxlin and eosin stain (H&E) and Masson's Trichrome stain) four weeks after injury. The numbers of the centronucleated regenerating myofibers from each group was determined to evaluate the regeneration. Northern Eclipse software (Empix Image, Inc.) was used to quantify the total fibrotic area. Statistical analysis was performed with ANOVA.

RESULTS:

Suramin stimulates MDSCs's differentiation: Suramin treatment promoted the differentiation of MDSCs *in vitro* in a dose dependent manner. We observed a significantly higher fusion index in each of the two suramin treatment groups (10 and 100μg/ml) than in the control group (0μg/ml). Furthermore, 100μg/ml of suramin treatment enhanced the differentiation significantly more than the other suramin treatments (1 and 10μg/ml) (Fig.1,2).

Suramin enhances muscle regeneration and decreases fibrosis after contusion injury: We observed a significant increase in the number of regenerating myofibers in all of suramin treated groups (2.5, 5, 10mg/20μl PBS) when compared with the control group (0mg/20μl of PBS) (Fig. 3,4). Moreover Masson's Trichrome staining showed significantly less fibrotic area in all of suramin treated groups than in the control group (Fig. 5, 6). Although all three suramin treated groups showed significant improvement in healing by way of muscle regeneration and fibrosis inhibition, there was no significant difference between the three suramin treatment groups (data not shown).

Figure 1. Immunocytochemistry of MyHC

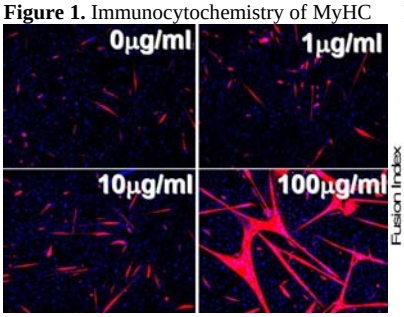


Figure 2. Fusion Index

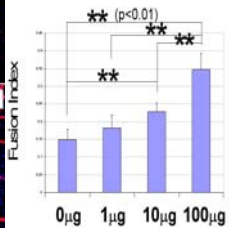


Figure 3. H-E staining 4 wks after contusion

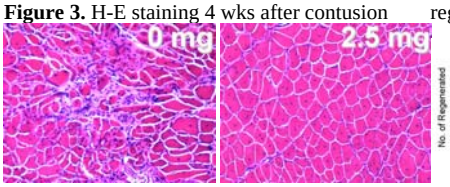


Figure 4. Number of regenerated myofibers

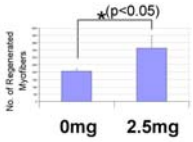


Figure 5. Trichrome staining after contusion

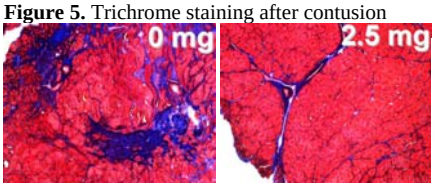
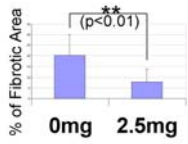


Figure 6. Percentage of Fibrotic area



DISCUSSION:

We have reported that suramin can effectively prevent muscle fibrosis and enhance muscle regeneration after laceration and strain injury. Furthermore, our preliminary data has indicated that suramin blocks the proliferative effect of TGF-β1 on fibroblasts *in vitro* [2,3]. We examined the effect of suramin on the differentiation of MDSCs after we observed that suramin enhanced the differentiation of C2C12 as a preliminary study. Our results from this experiment showed that the suramin treated MDSC groups have higher fusion indices than the control group in vitro. This indicates that suramin can enhance the differentiation of MDSCs, revealing part of the mechanism by which suramin enhances the muscle regeneration after injury. This is the first study to show that suramin is affecting the differentiation of MDSCs directly in addition to it's antiproliferative effect on fibroblasts. Furthermore, our result shows suramin can enhance muscle regeneration and prevent fibrosis after a contusion injury, the most common muscle injury. Our future study will investigate the mechanism by which suramin enhances the differentiation of myogenic cells.

Acknowledgements:

The authors are grateful for technical assistance from Maria Branca, Jessica Tebbets, and Aiping Lu. Funding support was provided by a grant from the Department of Defense (W81XWH-06-1-0406).

References:

1. Li Y, Foster W *et al., Am J Pathol* 2004;164: 1007-19.
2.Chan Y, Li Y *et al., J Appl Physiol* 2003;95:771-80.
3. Chan Y, Li Y *et al., Am J Sports Med* 2005; 33:43-51.
4.Qu-Peterson Z, *et al., J Cell Bio* 2002; 157: 851-64.

**Department of Orthopaedic Surgery, University of Pittsburgh

Appendix 2

Decorin Gene Transfer Promotes Muscle Cell Differentiation and Muscle Regeneration

Yong Li[1,2,3], Juan Li[2], Jinghong Zhu[1], Bin Sun[1], Maria Branca[1], Ying Tang[1], William Foster[1], Xiao Xiao[2] and Johnny Huard[1,2,4]

[1]*Stem Cell Research Center, Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania, USA;* [2]*Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania, USA;* [3]*Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA;* [4]*Department of Molecular Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, Pennsylvania, USA*

We have shown that decorin, a small leucine-rich proteoglycan, can inhibit transforming growth factor (TGF)-β1 to prevent fibrous scar formation and improve muscle healing after injury. In the decorin-treated muscle, an enhancement of muscle regeneration is observed through histological examination. In this article, we report our determination of whether decorin has a direct effect on myogenic cells' differentiation. Our results indicate that myoblasts genetically engineered to express decorin (CD cells) differentiated into myotubes at a significantly higher rate than did control myoblasts (C2C12). This enhanced differentiation led to the up-regulation of myogenic genes (*Myf5*, *Myf6*, *MyoD*, and myogenin) in CD cells in vitro. We speculate that the higher rate of differentiation exhibited by the CD cells is due to the up-regulation of follistatin, peroxisome-proliferator-activated receptor-gamma co-activator-1α (PGC-1α), p21, and the myogenic genes, and the down-regulation of TGF-β1 and myostatin. Decorin gene transfer *in vivo* promoted skeletal muscle regeneration and accelerated muscle healing after injury. These results suggest that decorin not only prevents fibrosis but also improves muscle regeneration and repair.

Received 13 November 2006; accepted 20 May 2007; published online 3 July 2007. doi:10.1038/sj.mt.6300250

INTRODUCTION

Decorin, a small leucine-rich proteoglycan, is a component of the extracellular matrix of all collagen-containing tissues.[1] Decorin is pivotal in regulating the proper assembly of collagenous matrices and in controlling cell proliferation under various conditions.[2] On the basis of its ability to bind fibrillar collagen and delay *in vitro* fibrillogenesis, decorin is regarded as a key modulator of matrix assembly.[3,4] This proteoglycan can modulate the bioactivity of growth factors and act as a direct signaling molecule to different cells.[5] Decorin, which is expressed at high levels in skeletal muscle during early development,[6] also interferes with muscle cell differentiation and migration and regulates connective tissue formation in skeletal muscle.[7–9]

Because terminal differentiation is critical for initial skeletal muscle development and regeneration after injury and disease,[10] we examined decorin's role in remodeling healing skeletal muscle. We have shown that the direct injection of bovine decorin decreased muscle fibrosis and provided nearly complete functional recovery.[11] Decorin blocks fibrosis (mostly by inhibiting transforming growth factor (TGF)-β activity), which improves muscle healing. However, the role of decorin in muscle cell differentiation and regeneration is still unknown. Although we hypothesize that decorin's effect on muscle fibrosis may indirectly impact regeneration, we were unable to exclude the possibility that decorin promotes regeneration independent of its effects on fibrosis formation.

Many studies have investigated the mechanism behind the antifibrotic effect of decorin.[3,12–14] Others have shown that hepatocyte growth factor increases decorin production by fibroblasts through the extracellular signal–regulated kinase 1/2, and p38 mitogen-activated protein kinase–mediated pathways.[14] Decorin stimulates the growth of smooth muscle cells under specific conditions and influences the growth of epidermal cells by interacting with epidermal growth factor and its receptors.[15,16] Recent research has shown that decorin can bind both insulin-like growth factor-I and its receptor; this interaction leads to the phosphorylation of protein kinase B (Akt) and p21 expression in endothelial cells.[17]

Decorin also influences muscle cell behavior by interacting with p21, an important cyclin-dependent kinase inhibitor.[18,19] Follistatin and myostatin are involved in the control of muscle mass during development. These two proteins have opposite effects on muscle growth, as documented by genetic models.[21,22] Recent studies have shown that myostatin action is inhibited by decorin,[23] resulting in enhanced healing and reduced fibrosis within myostatin-null mice compared with wild-type mice.[24] A recent study indicates that peroxisome-proliferator-activated receptor-gamma co-activator-1α (PGC-1α), is also involved in the muscle healing process and influences muscle fiber–type determination.[25,26] Decorin may also interact with PGC-1α expression in skeletal muscle after injury.

In this study, we investigated the *in vitro* effect of decorin on the differentiation of myoblasts (C2C12) and characterized the *in vitro* and *in vivo* behavior of myoblasts transfected with the

Correspondence: *Johnny Huard, Stem Cell Research Center (SCRC), 4100 Rangos Research Center, 3460 Fifth Avenue, Pittsburgh, Pennsylvania 15213-2583, USA. E-mail: jhuard@pitt.edu*

decorin gene (CD cells). We also studied the influence that decorin over-expression had on myostatin, follistatin, PGC-1α, and p21 expression. Using an adeno-associated virus (AAV) vector, we transduced the decorin gene into injured skeletal muscle to further investigate its function on muscle healing. Our overall goal in this study was to determine whether decorin could improve skeletal muscle healing by enhancing muscle regeneration independently of its antifibrotic action.

RESULTS

Genetic engineering of myogenic cells to over-express decorin

We used lipofectin to transfect a pAAV-CMV-decorin plasmid (**Figure 1a**) into both 293 cells (packaging cell line) and C2C12 cells (myoblast cell line). The results of western blot analysis (**Figure 1b**) showed decorin in both the supernatant (culture media) and the lysate of the 293 cells 48 hours after transfection. The transfected C2C12 cells (CD clone cells) expressed decorin (**Figure 1c:** lane 2, 24 hours; lane 4, 48 hours; lane 5, decorin-positive control), whereas non-transfected C2C12 cells did not (**Figure 1c:** lane 1, 24 hours; lane 3, 48 hours). We also detected decorin in both myoblasts (C2C12) and muscle-derived stem cells after mDecorin-AAV (mDec-AAV) gene transfer *in vitro* (**Figure 1d** and **e**).

Decorin stimulates myoblast differentiation *in vitro*

To investigate myoblast differentiation, we compared decorin-co-cultured C2C12 cells with non-treated C2C12 cells *in vitro*. C2C12 cells cultured with decorin (10 μg/mL) and grown in differentiation/fusion medium exhibited significantly enhanced differentiation and fusion *in vitro*. After 3 and 4 days of stimulating C2C12 cells with decorin, we observed a significant increase in the number of myotubes when compared with un-stimulated C2C12 (control) cells ($P < 0.01$ at 3 days and $P < 0.05$ at 4 days, respectively). However, the numbers of myotubes 5 days after treatment were not significantly different (**Figure 2a**). We then evaluated whether CD cells exhibited a greater propensity to undergo myogenic differentiation than did non-transfected C2C12 cells. As shown in **Figure 2b** (myotubes stained in mouse anti-myosin heavy chain are red) and **Figure 2c**, CD cells generated significantly more myotubes overall and created significantly larger myotubes than did non-transfected C2C12 cells.

Decorin increases myoblast differentiation and induces myogenic gene expression *in vitro*

We investigated whether the CD cells expressed higher levels of myogenic genes than did non-transfected C2C12 (control) cells. Our results, shown in **Figure 3a**, demonstrate that decorin gene transfer led to higher expression of the myogenic genes *Myf5*, *Myf6*, *MyoD*, and *myogenin*. Desmin expression levels in CD and C2C12 cells remained similar.

Decorin up-regulates p21, follistatin, and PGC-1α, but down-regulates TGF-β1 and myostatin in C2C12

We also performed experiments designed to investigate the mechanism by which decorin influences the differentiation of muscle cells. We found that CD cells exhibited increased p21 expression and decreased myostatin expression (**Figure 3b**). The C2C12 cells can be induced to express TGF-β1 in an autocrine manner, as we have previously determined;[27] however, the CD cells do not show any detectable expression of TGF-β1 after

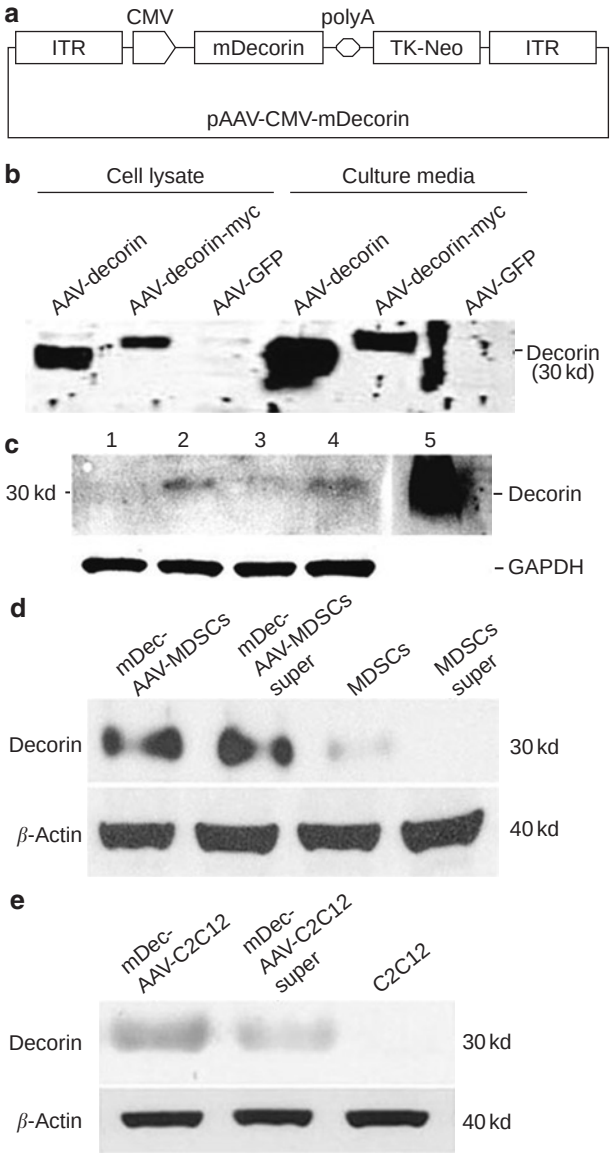


Figure 1 Decorin plasmid construction and initial transfection *in vitro*. (**a**) The decorin plasmid used for the study contained the full sequence of a mouse decorin gene inserted at the *Not*I site, which placed it under the control of a cytomegalovirus (CMV) promoter. (**b**) We transfected the plasmid into 293 cells. We observed decorin expression in both the 293 cells and their supernatant, but not in the control adeno-associated virus (AAV)–transfected (green fluorescent protein, GFP) cells. (**c**) Western blot analysis also revealed decorin expression in CD clone cells within different time period cultures (lane 2, 24 hours; lane 4, 48 hours), but not in C2C12 (lane 1, 24 hours; lane 3, 48 hours). We used 5 μg of decorin as a positive control (lane 5). GAPDH, glyceraldehyde-3-phosphate dehydrogenase is used as a control. (**d**) Decorin expression in muscle-derived stem cells (MDSCs) in pellet form was low, but this was not the case in the supernatant. Both MDSCs and their cultured supernatant strongly expressed decorin after mDec-AAV gene transfer. β-actin is used as a control. (**e**) We did not detect decorin in normal C2C12 cells, but C2C12 cells and their cultured supernatant both expressed decorin after mDec-AAV gene transfer.

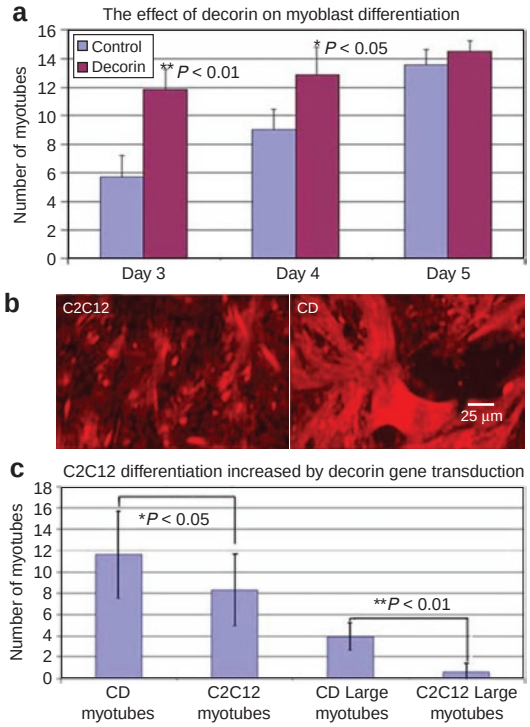


Figure 2 Decorin stimulates C2C12 differentiation *in vitro*. (**a**) Decorin treatment accelerated the differentiation and fusion of myoblasts (C2C12 cells) compared with non-treated myoblasts (C2C12 cells). (**a**) The cultures of decorin-treated C2C12 cells contained more myotubes at the 3- and 4-day time points than control cells. (**b**, **c**) Similarly, decorin-transfected C2C12 clone cells (CD cells) produced more myotubes than did C2C12 cells, including larger myotubes (containing more than three nuclei) *in vitro*. Red staining shows myosin heavy chain fluorescence after immunostaining (**b**).

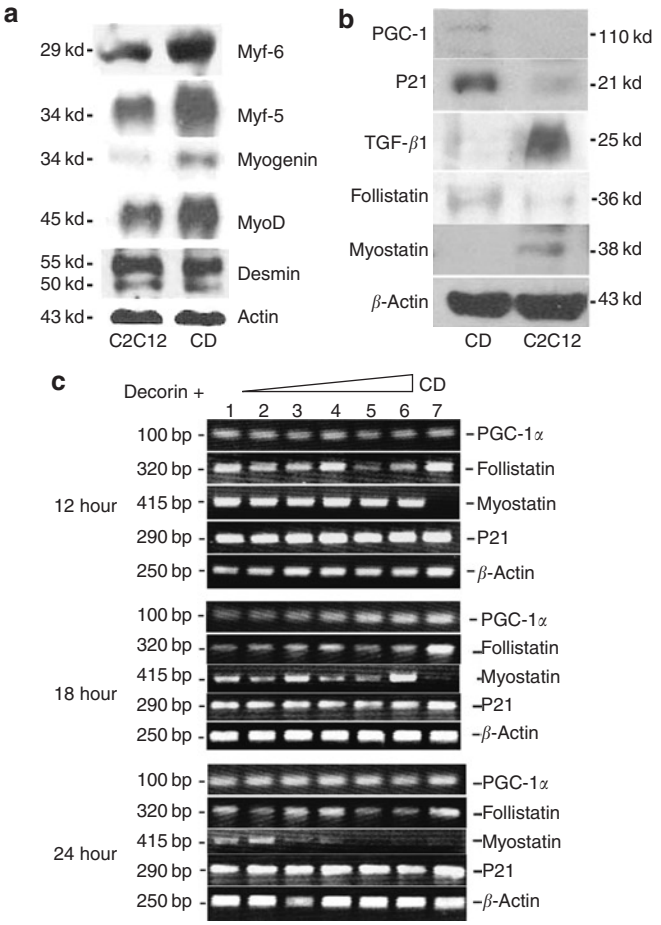


Figure 3 Decorin gene transfer up-regulates myogenic proteins and p21 and down-regulates myostatin during muscle cell differentiation. (**a**) Genetic engineering of myoblasts to express decorin influenced the expression of myogenic proteins (including Myf5/6, Myogenin, and MyoD), as shown by western blot results. However, C2C12 cells and CD clone cells expressed comparable levels of desmin. (**b**) We detected the presence of p21 expressed in CD clone cells. We also detected peroxisome-proliferator-activated receptor-gamma co-activator-1α (PGC-1α) and follistatin expressed in CD, but not C2C12, cells. In addition, CD cells exhibited lower levels of myostatin, a negative regulator of muscle mass. The induction of tumor growth factor (TGF)-β1 auto-expression was also inhibited by decorin over-expression in CD cells. (**c**) Similar results were obtained by real-time polymerase chain reaction. Lanes 1–6 show results for C2C12 cells exposed to different concentrations of decorin (0, 0.001, 0.01, 0.1, 1.0, and 5.0 ng/ml, respectively). Lane 7 displays the test results for CD cells, which served as the positive control. We did not detect a visible change in the expression of PGC-1α, follistatin, myostatin, or p21 after 12 hours of stimulation with different concentrations of decorin. PGC-1α and follistatin were up-regulated in C2C12 cells in a dose-dependent manner after 18 hours of stimulation with decorin. Myostatin was down-regulated in C2C12 cells in a dose-dependent manner after 24 hours of decorin stimulation. The concentration of p21 did not visibly change after cell stimulation with any experimental concentration of decorin over all time points. With decorin gene transfer, we found that CD cells consistently expressed follistatin, p21, and PGC-1α but were negative for myostatin. Note that β-actin was selected as a positive gene control.

TGF-β1 stimulation. More importantly, we detected that both follistatin and PGC-1α (**Figure 3b**) had been up-regulated when compared with C2C12 cells. We also discovered that follistatin and PGC-1α messenger RNA were altered in C2C12 cells after decorin stimulation, as determined by real-time polymerase chain reaction. Specifically, we found that PGC-1α and follistatin increased in a dose-dependent manner after 18 hours of stimulation with decorin, and that myostatin was decreased in a dose-dependent manner after 24 hours of stimulation with decorin (**Figure 3c**). In CD cells, we also observed increased amounts of all three genes (*p21*, *follistatin*, and *PGC-1α*), but a decrease in myostatin was observed (**Figure 3c**).

The up-regulation of *follistatin*, *PGC-1α*, *p21*, and myogenic genes, including *MyoD* (**Figure 3a**), in CD cells could at least partially explain how decorin promotes muscle cell differentiation. Alternatively, the down-regulation of myostatin, a well-known negative regulator of muscle growth during muscle regeneration, could also benefit muscle cell differentiation.

The implantation of CD cells in skeletal muscle results in improved muscle regeneration

The implantation of CD cells within skeletal muscle resulted in significantly better muscle regeneration than that observed for control C2C12 cells, as determined 4 weeks after injection of the cells into *MDX*/SCID mice. Although the number of LacZ-positive muscle fibers (*i.e.*, regenerating muscle fibers) did not differ between the groups (**Figure 4a**, **c**, and **d**), the diameters of the regenerating muscle fibers (*e.g.*, dystrophin-positive myofibers) in the muscles injected with CD cells were significantly larger than those of the regenerating muscle fibers in the control muscles

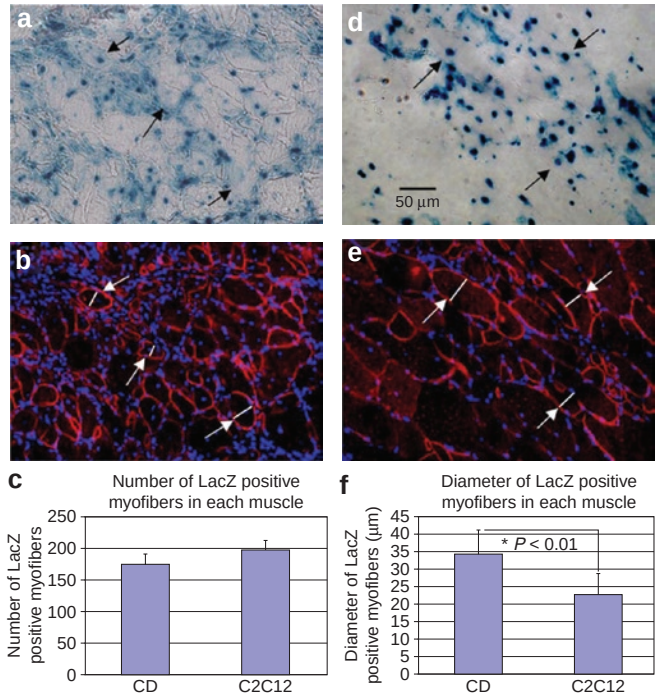


Figure 4 Decorin gene transfer stimulates muscle regeneration *in vivo*. C2C12 cells regenerated muscle fibers after transplantation into skeletal muscle of *MDX*/SCID mice, as shown on both (**a**) LacZ- and (**b**) dystrophin-expressing myofibers. However, transplantation of CD clone cells, rather than C2C12 cells, resulted in larger muscle fibers in *MDX*/SCID mice, as shown in some of the (**d**) LacZ- and (**e**) dystrophin-positive myofibers. (**c**) Although there was no significant difference between the number of LacZ-labeled muscle fibers that formed in muscles transplanted with C2C12 or CD cells, (**f**) the transplantation of CD cells resulted in the regeneration of larger-diameter myofibers ($P < 0.01$).

(**Figure 4b**, **e**, and **f**; dystrophin is red). The larger diameters of the dystrophin-positive muscle fibers generated by CD cells could indicate that implantation of CD cells accelerated muscle regeneration; however, we were unable to exclude the possibility that the CD cells may have a greater propensity to fuse in host myofibers than C2C12 control cells.

mDec-AAV gene transfer promotes muscle regeneration and reduces fibrosis

Better muscle regeneration was observed within mDec-AAV-treated muscle (**Figure 5a** and **b**) than within non-mDec-AAV-treated muscle at 2 weeks after injury (**Figure 5c** and **d**). Histological analysis of total collagen deposition 4 weeks after injury revealed that mDec-AAV-injected muscles contained less fibrous scar tissue in the injured area than did non-treated control muscles (**Figure 5f**, **h**, and **j**; collagen deposition areas are blue). We also observed that decorin stimulated skeletal muscle regeneration 4 weeks after laceration injury. We found that mDec-AAV-injected muscles contained more centronucleated (regenerating) myofibers and less scar tissue 4 weeks after injury than did control muscles (**Figure 5e**, **g**, and **i**).

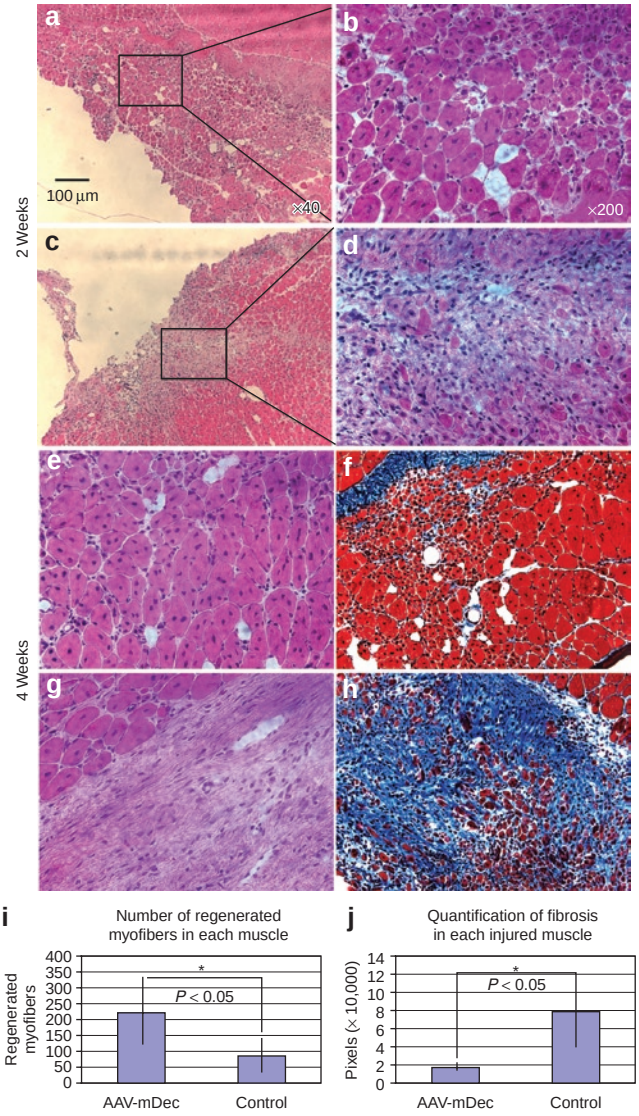


Figure 5 mDec-AAV vector gene therapy in injured muscle prevents fibrosis and promotes muscle regeneration. Decorin-treated muscle exhibits a greater number of regenerating myofibers than the control muscle at all time points (**a–d** 2 weeks; **e–h** 4 weeks). (**i**) The mDec-AAV-injected muscle contained significantly higher numbers of centronucleated (regenerated) myofibers than did control (sham-injected) muscle at 4 weeks after therapy. We also found that decorin gene therapy minimized fibrosis in injured skeletal muscle. We used Masson's trichrome staining to reveal collagen in injured skeletal muscle, the results of which show that (**f**) mDec-AAV-injected muscle contained significantly less fibrosis in the injured area than did the (**h**) control muscle at (**j**) 4 weeks after injury.

DISCUSSION

Results from these experiments show that decorin is able to activate the differentiation of skeletal muscle cells (C2C12) *in vitro* and enhances muscle regeneration in two mouse models *in vivo*. The mechanism behind decorin's accelerated muscle healing is not yet known; however, our results demonstrate that decorin up-regulates the expression of PGC-1α, follistatin, p21, and a variety of myogenic proteins (including MyoD) but down-regulates myostatin expression. These results, in addition to decorin's ability to neutralize the effects of TGF-β1, likely explain the beneficial action that decorin has on muscle cell differentiation and muscle regeneration.

Our previous studies have demonstrated that myogenic cells (including muscle-derived stem cells) in injured muscle can

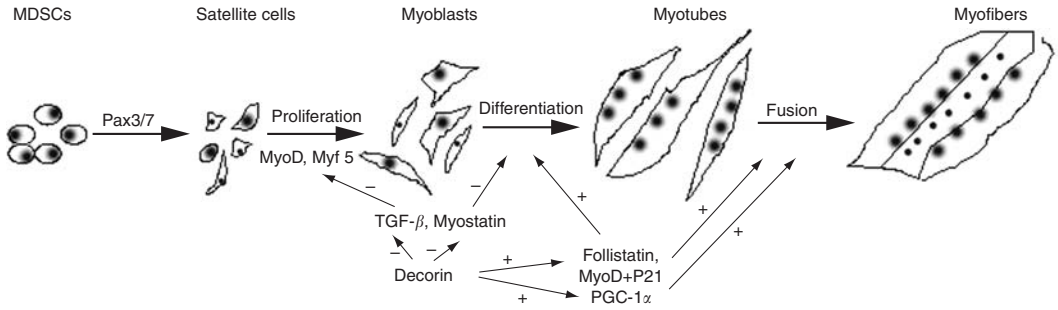


Figure 6 Schematic of the potential effect of decorin on muscle healing. Decorin may improve muscle healing through various pathways: inhibition of tumor growth factor (TGF)-β1, up-regulation of follistatin, peroxisome-proliferator-activated receptor-gamma co-activator-1α (PGC-1α), p21, and myogenic genes (such as *MyoD*), and down-regulation of myostatin expression. MDSCs, muscle-derived stem cells.

differentiate into fibrotic cells and that TGF-β1 is a major stimulator of this differentiation.[27,28] Using different animal models of muscle injury, we have investigated biological approaches to prevent fibrosis and thereby improve muscle healing.[11,27,29–31] We have used various molecules, such as decorin, that impede fibrosis by blocking TGF-β1 to facilitate the near-complete recovery of injured skeletal muscle.[11] The ability of decorin to inhibit TGF-β1 activity is the likely mechanism by which this molecule blocks fibrosis formation. However, our results indicate that the improved muscle healing observed after decorin treatment is due to both its inhibiting effect on fibrosis and its stimulating effect on muscle regeneration. **Figure 6** summarizes the potential effect of decorin on muscle healing.

The repair of injured skeletal muscle occurs through the activation of muscle precursor cells located between the basal lamina and the sarcolemma, including satellite cells and stem cells.[32] The activation and growth of these cells are regulated by various growth factors released by infiltrating lymphocytes, injured myofibers, and the extracellular matrix.[10,32] Some growth factors, such as insulin-like growth factor-1 and hepatocyte growth factor, can stimulate precursor cell proliferation and differentiation by increasing the transcriptional activity of the muscle basic helix–loop–helix.[33–36] Healing and organizational processes are dependent upon the extra- and intracellular signaling that induces the expression of myogenic genes, including *MyoD*, *Myf5*, and *myosin*.[37,38] When properly stimulated, precursor cells fuse with one another or with local myofibers to repair the damaged muscle.[39]

Muscle regeneration, the key event in muscle healing, is often incomplete, particularly in severely injured muscle.[10,11,27,28,32,40] The overgrowth of the extracellular matrix leads to significant local fibrosis (*i.e.*, fibrous scar formation) in the injured area, which can impede the formation of normal muscle fibers. The presence of fibrous scar tissue in injured muscle results in incomplete functional recovery and a propensity for re-injury.[41] Muscle regeneration and fibrosis in injured muscle often occur simultaneously and thus compete with one another during the muscle healing process.[32,36,40] A persistent imbalance between collagen biosynthesis and degradation contributes to hypertrophic scar formation and fibrosis in many tissues.[42,43] Several studies have revealed high levels of collagen in injured regions of skeletal muscle, and shown that inhibition of collagen deposition reduced the formation of scar tissue in injured skeletal muscle.[27,28,30,31]

Interactions between decorin and TGF-β1 have been observed in many tissues, and researchers have used various animal models to study the antifibrotic effect of decorin.[3,11,13,44] Researchers have also shown that hepatocyte growth factor can increase the level of decorin expression in fibroblasts, perhaps by activating the extracellular signal–regulated kinase 1/2 and p38 mitogen-activated protein kinase–mediated pathways.[14] Such findings could explain the antifibrotic effect of hepatocyte growth factor in a variety of tissues.[14,45–47] In endothelial cells, decorin binds with both insulin-like growth factor-I and its receptor to influence cell behavior.[17] Decorin can also control and suppress cancer growth and invasion, presumably by influencing the biological activity of growth factors such as TGF-β1, platelet-derived growth factor, vascular endothelial growth factor, and epidermal growth factor, all of which are released by cancer cells.[5,15] These decorin-induced effects appear to be mediated, at least in part, by a specific interaction between the decorin protein core and the epidermal growth factor receptor.[15,16] This interaction triggers a signal cascade that results in activation of mitogen-activated protein kinase, mobilization of intracellular calcium, up-regulation of p21, and, ultimately, the suppression of tumor growth.[18,19]

Cell cycle exit and the differentiation of muscle cells are coordinated by p21, which is essential for normal myogenic progenitor cell differentiation and skeletal muscle regeneration. Studies have indicated that p21 is necessary for MyoD-induced activity in cells, allowing them to enter into and be stabilized in a postmitotic state. Since MyoD plays a central role in the differentiation of muscle cells, TGF-β1 controls myostatin-related regulation of myogenesis in muscle cells by down-regulating both p21 and MyoD. In this study, we determined that the treatment of myoblasts with decorin down-regulated the expression of myostatin, which might influence p21 and myogenic protein expression. In addition, myostatin and follistatin interact directly in the skeletal muscle system. Follistatin can inhibit myostatin, leading to muscle differentiation in a concentration-dependent manner.[21] PGC-1α, which is expressed in several tissues, including brown fat and the skeletal muscle of mammals, activates mitochondrial biogenesis and oxidative metabolism.[26] PGC-1α is a principal factor involved in determining muscle fiber type in injured skeletal muscle and is involved in exercise-induced mitochondrial biogenesis.[48] In this experiment, we observed that decorin treatment increased PGC-1α expression in skeletal muscle cells. Combined with our previous research results, our current findings suggest that decorin not

only acts as an antifibrotic agent but also enhances muscle regeneration in skeletal muscle.

Successful muscle differentiation during limb development requires decorin expression.[6] Previous findings have shown that decorin can improve muscle healing by inhibiting fibrosis and that myoblasts and muscle satellite cells expressing decorin in an injured site regenerated damaged myofibers faster than the controls.[11,27] The results of this study demonstrate that decorin is also a potent stimulator of skeletal muscle regeneration. Myoblasts expressing decorin differentiated and fused to form myotubes and myofibers at a significantly higher rate than did normal myoblasts *in vitro* and *in vivo*. We attribute this enhanced differentiation to the up-regulation of p21, follistatin, PGC-1α, and myogenic gene expression and the down-regulation of TGF-β1 and myostatin. These results provide at least a partial explanation of the way in which decorin promotes muscle regeneration and may explain why there is such a high level of decorin expression in developing skeletal muscle. It is possible that decorin increases muscle fiber growth and limits the overgrowth of connective tissues. These findings indicate that decorin could be very useful in promoting the healing of muscles damaged by injury or disease.

MATERIALS AND METHODS

Gene transfection and transfer. An AAV-mDecorin plasmid, which encodes for a mouse decorin sequence under the control of the cytomegalovirus promoter (**Figure 1**), was used for gene transfection. This plasmid also contains a neomycin resistance gene to enable G418 selection. The AAV-mDecorin plasmid was transfected into 293 packaging cells and C2C12 cells with lipofectin; clone cells were selected for treatment of the cells with G418 (500 μg/mL) (Gibco BRL, Grand Island, NY) for 2 weeks. The selected decorin-transfected C2C12 clone cells (CD cells) were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) containing the same concentration of G418 for the remainder of the project.

The mDec-AAV vector was produced by co-transfection methods described previously by Dr. Xiao.[49] Muscle-derived stem cells and myoblasts (C2C12 cells) were each grown to 50–60% confluency. Fresh Dulbecco's modified Eagle's medium (without fetal bovine serum or penicillin/streptomycin) containing the mDec-AAV vector (5×10^4 particles/cell) was then added directly to the cells. The cultures were incubated at 37 °C in a 5% CO_2 incubator for 1 hour. Normal culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% Abs) was then added for another 24 hours, at which point the cells were collected for analysis of decorin expression by western blotting.

Differentiation of myoblasts and immunocytochemistry. Three different groups of cells (C2C12 cells, C2C12 cells cultured with decorin, and CD cells) were seeded into 12-well plates containing proliferation medium.[28] All cells were transferred into serum-free medium 12 hours later to induce differentiation. The myotubes that formed in the cultures were counted daily for 5 days, and the numbers were compared among the groups. We considered myotubes containing three or more nuclei to be large myotubes *in vitro*. At different time points, the cells were fixed with cold acetone (3 minutes) for immunostaining. Mouse anti-myosin heavy chain antibody (Novocastra Lab) at a 1:200 dilution was applied for 1 hour at room temperature (RT). The primary antibody was detected using anti-mouse-Cy3, 1:250 for 45 minutes at RT. Results were analyzed by fluorescent microscopy (Nikon microscope, Nikon, Melville, New York).

Real-time polymerase chain reaction. Total RNA was extracted from the treated and non-treated C2C12 cells using a Nucleospin column (Clontech, Mountan View, CA), and the complementary DNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), both according to manufacturer's instructions. Primers specific for *myostatin*, *follistatin*, *p21*, and *PGC-1α* were designed using Oligo software (OligoPerfect Designer; Invitrogen, Carlsbad, CA). The protocol for amplification was as follows: 94 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds for 30 cycles. Polymerase chain reaction products were separated by size in a 1.5% agarose gel.

Western blot analysis. C2C12 and CD cells were lysed when cell density reached 70% confluency. The samples were separated on a 12% sodium dodecyl sulfate–polyacrylamide electrophoresis gel and transferred to nitrocellulose membranes used to perform immunostaining. The primary antibodies were anti-decorin (a gift from Dr. Fisher of the National Institutes of Health), anti-TGF-β1 (4 μg/mL; BD Pharmingen, San Diego, CA), anti-p21 (BD Pharmingen, San Diego, CA), anti-myf5, anti-myf6, anti-MyoD, and anti-myogenin (Santa Cruz Bio, Santa Cruz, CA), all at concentrations of 1:1,000, and anti-myostatin, anti-follistatin (Chemicon, Temecula, CA), and anti-desmin (Sigma, St. Louis, MO), all at concentrations of 1:2,000 for 1 hour at RT. Mouse anti-β-actin and anti-glyceraldehyde-3-phosphate dehydrogenase (Sigma, St. Louis, MO) were used for protein quantification and were diluted to 1:8,000. The secondary anti-rat horseradish peroxidase or anti-rabbit horseradish peroxidase (Pierce, Rockford, IL) was used at a concentration of 1:5,000 for 1 hour. Peroxidase activity was determined by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ), and the positive bands were detected on X-ray film. Northern Eclipse software v.6.0 (Empix Imaging, Mississauga, Canada) was used to evaluate all results.

Animal experiments. All animal experiments were approved by the Children's Hospital of Pittsburgh. The Animal Research Committee at the authors' institution approved all experimental protocols (No. 15/03).

Group 1: C2C12 and CD cell transplantation. Twenty-four female *MDX*/SCID mice (C57BL/10ScSn-*Dmd*^{mdx} crossed with C57BL/6J-*Prkdc*^{scid}/SzJ, 6–8 weeks of age) were used for the C2C12 and CD cell transplantation. C2C12 and CD clone cells were transduced with a retrovirus vector encoding for LacZ.[27] LacZ-positive CD cells (1×10^6) were injected into the left gastrocnemius muscles (GMs); the same quantity of LacZ-positive C2C12 cells were injected into the right GMs as a control. At various times after injection, mice were killed, and the GMs were collected for histological analysis by LacZ staining and immunohistochemistry to stain for dystrophin-positive myofibers.

Group 2: mDec-AAV gene therapy administered to injured skeletal muscle. Twenty mice (C57BL6J$^{+/+}$, 6 weeks old; Jackson Laboratory, Bar Harbor, ME) were used for these experiments. The mDec-AAV vector (2×10^{11} particles in 20 μL of Dulbecco's modified Eagle's medium) was injected directly into the left GM of each mouse; the contralateral leg was injected with the same volume of phosphate-buffered saline (20 μL) as a control. One week after injection, both GMs were lacerated in accordance with our previously described muscle injury model.[11,27,28,40] Mice were killed at different time points (5 days and 1, 2, 3, and 4 weeks after injury), and the GMs were collected for histological analysis by either hematoxylin and eosin or Masson's trichrome staining. The regeneration and fibrous scar tissue formation in the two groups were compared.

Immunohistochemical analysis. Serial 10-μm cryostat sections were prepared using standard techniques.[27,28] For immunohistochemistry, the slides were fixed with formalin (4%) for 5 minutes after LacZ staining, and then blocked with donkey serum (10%) for 1 hour. Rabbit anti-dystrophin antibody (Abcam, Cambridge, MA) was applied to the slides at a 1:300 dilution for 60 minutes at RT. The second antibody, goat anti-rabbit IgG (Alexa Fluor® 488; Molecular Probes, Eugene, OR), was used at a concentration of 1:200 for 45 minutes at RT. Negative controls were performed concurrently with all immunohistochemical staining. The nuclei of the sections were revealed using 4′,6′-diamidino-2-phenylindole hydrochloride

staining (Sigma, St. Louis, MO), and fluorescent microscopy was used to visualize the results as described above.

Statistical analysis. LacZ-positive myofibers were counted in 10 representative sections. Both the diameter and number of LacZ- and dystrophin-positive myofibers were assessed at different time points in each group. The statistical significance of differences between the various groups was determined using a *t*-test or one-way or two-way analysis of variance.

ACKNOWLEDGMENTS

The authors wish to thank James Cummins, Marcelle Pellerin, and Jing Zhou (Stem Cell Research Center, Children's Hospital of Pittsburgh, Pittsburgh, PA) for their technical assistance, Paul Robbins (Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA) for his contribution of the LacZ retrovirus vector, and Ryan Sauder, Shannon Bushyeager, and David Humiston (Stem Cell Research Center) for their excellent editorial assistance with this manuscript. The authors also gratefully acknowledge the financial support of the Department of Defense in the form of grant W81XWH-06-01-0406 and the National Institutes of Health in the form of NIH grant R01 AR47973.

REFERENCES

1. Hocking, AM, Shinomura, T and McQuillan, DJ (1998). Leucine-rich repeat glycoproteins of the extracellular matrix. *Matrix Biol* **17**: 1–19.
2. Sottile, J, Hocking, DC and Swiatek, PJ (1998). Fibronectin matrix assembly enhances adhesion-dependent cell growth. *J Cell Sci* **111**: 2933–2943.
3. Giri, SN, Hyde, DM, Braun, RK, Gaarde, W, Harper, JR and Pierschbacher, MD (1997). Antifibrotic effect of decorin in a bleomycin hamster model of lung fibrosis. *Biochem Pharmacol* **54**: 1205–1216.
4. Noble, NA, Harper, JR and Border, WA (1992). *In vivo* interactions of TGF-beta and extracellular matrix. *Prog Growth Factor Res* **4**: 369–382.
5. Stander, M, Naumann, U, Dumitrescu, L, Heneka, M, Loschmann, P, Gulbins, E *et al.* (1998). Decorin gene transfer-mediated suppression of TGF-beta synthesis abrogates experimental malignant glioma growth *in vivo*. *Gene Ther* **5**: 1187–1194.
6. Nishimura, T, Futami, E, Taneichi, A, Mori, T and Hattori, A (2002). Decorin expression during development of bovine skeletal muscle and its role in morphogenesis of the intramuscular connective tissue. *Cells Tissues Organs* **171**: 199–214.
7. Brandan, E, Fuentes, ME and Andrade, W (1991). The proteoglycan decorin is synthesized and secreted by differentiated myotubes. *Eur J Cell Biol* **55**: 209–216.
8. Casar, JC, McKechnie, BA, Fallon, JR, Young, MF and Brandan, E (2004). Transient up-regulation of biglycan during skeletal muscle regeneration: delayed fiber growth along with decorin increase in biglycan-deficient mice. *Dev Biol* **268**: 358–371.
9. Yoshida, N, Yoshida, S, Koishi, K, Masuda, K and Nabeshima, Y (1998). Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. *J Cell Sci* **111**: 769–779.
10. Li, Y, Cummins, J and Huard, J (2001). Muscle injury and repair. *Curr Opin Orthop* **12**: 409–415.
11. Fukushima, K, Badlani, N, Usas, A, Riano, F, Fu, F and Huard, J (2001). The use of an antifibrosis agent to improve muscle recovery after laceration. *Am J Sports Med* **29**: 394–402.
12. Harper, JR, Spiro, RC, Gaarde, WA, Tamura, RN, Pierschbacher, MD, Noble, NA *et al.* (1994). Role of transforming growth factor beta and decorin in controlling fibrosis. *Methods Enzymol* **245**: 241–254.
13. Isaka, Y, Brees, DK, Ikegaya, K, Kaneda, Y, Imai, E, Noble, NA *et al.* (1996). Gene therapy by skeletal muscle expression of decorin prevents fibrotic disease in rat kidney. *Nat Med* **2**: 418–423.
14. Kobayashi, E, Sasamura, H, Mifune, M, Shimizu-Hirota, R, Kuroda, M, Hayashi, M *et al.* (2003). Hepatocyte growth factor regulates proteoglycan synthesis in interstitial fibroblasts. *Kidney Int* **64**: 1179–1188.
15. Csordas, G, Santra, M, Reed, CC, Eichstetter, I, McQuillan, DJ, Gross, D *et al.* (2000). Sustained down-regulation of the epidermal growth factor receptor by decorin. A mechanism for controlling tumor growth *in vivo*. *J Biol Chem* **275**: 32879–32887.
16. Iozzo, RV, Moscatello, DK, McQuillan, DJ and Eichstetter, I (1999). Decorin is a biological ligand for the epidermal growth factor receptor. *J Biol Chem* **274**: 4489–4492.
17. Schonherr, E, Sunderkotter, C, Iozzo, RV and Schaefer, L (2005). Decorin, a novel player in the insulin-like growth factor system. *J Biol Chem* **280**: 15767–15772.
18. De Luca, A, Santra, M, Baldi, A, Giordano, A and Iozzo, RV (1996). Decorin-induced growth suppression is associated with up-regulation of p21, an inhibitor of cyclin-dependent kinases. *J Biol Chem* **271**: 18961–18965.
19. Schonherr, E, Levkau, B, Schaefer, L, Kresse, H and Walsh, K (2001). Decorin-mediated signal transduction in endothelial cells. Involvement of Akt/protein kinase B in up-regulation of p21(WAF1/CIP1) but not p27(KIP1). *J Biol Chem* **276**: 40687–40692.
20. Budasz-Rwiderska, M, Jank, M and Motyl, T (2005). Transforming growth factor-beta1 upregulates myostatin expression in mouse C2C12 myoblasts. *J Physiol Pharmacol* **56** (suppl. 3): 195–214.
21. Amthor, H, Nicholas, G, McKinnell, I, Kemp, CF, Sharma, M, Kambadur, R *et al.* (2004). Follistatin complexes Myostatin and antagonises Myostatin-mediated inhibition of myogenesis. *Dev Biol* **270**: 19–30.
22. Lee, SJ and McPherron, AC (2001). Regulation of myostatin activity and muscle growth. *Proc Natl Acad Sci USA* **98**: 9306–9311.
23. Miura, T, Kishioka, Y, Wakamatsu, J, Hattori, A, Hennebry, A, Berry, CJ *et al.* (2006). Decorin binds myostatin and modulates its activity to muscle cells. *Biochem Biophys Res Commun* **340**: 675–680.
24. McCroskery, S, Thomas, M, Platt, L, Hennebry, A, Nishimura, T, McLeay, L *et al.* (2005). Improved muscle healing through enhanced regeneration and reduced fibrosis in myostatin-null mice. *J Cell Sci* **118**: 3531–3541.
25. Duguez, S, Feasson, L, Denis, C and Freyssenet, D (2002). Mitochondrial biogenesis during skeletal muscle regeneration. *Am J Physiol Endocrinol Metab* **282**: E802–E809.
26. Lin, J, Wu, H, Tarr, PT, Zhang, CY, Wu, Z, Boss, O *et al.* (2002). Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* **418**: 797–801.
27. Li, Y, Foster, W, Deasy, BM, Chan, Y, Prisk, V, Tang, Y *et al.* (2004). Transforming Growth Factor-beta1 Induces the Differentiation of Myogenic Cells into Fibrotic Cells in Injured Skeletal Muscle: A Key Event in Muscle Fibrogenesis. *Am J Pathol* **164**: 1007–1019.
28. Li, Y and Huard, J (2002). Differentiation of muscle-derived cells into myofibroblasts in injured skeletal muscle. *Am J Pathol* **161**: 895–907.
29. Chan, YS, Li, Y, Foster, W, Fu, FH and Huard, J (2005). The use of suramin, an antifibrotic agent, to improve muscle recovery after strain injury. *Am J Sports Med* **33**: 43–51.
30. Chan, YS, Li, Y, Foster, W, Horaguchi, T, Somogyi, G, Fu, FH *et al.* (2003). Antifibrotic effects of suramin in injured skeletal muscle after laceration. *J Appl Physiol* **95**: 771–780.
31. Sato, K, Li, Y, Foster, W, Fukushima, K, Badlani, N, Adachi, N *et al.* (2003). Improvement of muscle healing through enhancement of muscle regeneration and prevention of fibrosis. *Muscle Nerve* **28**: 365–372.
32. Huard, J, Li, Y and Fu, FH (2002). Muscle injuries and repair: current trends in research. *J Bone Joint Surg Am* **84-A**: 822–832.
33. Engert, JC, Berglund, EB and Rosenthal, N (1996). Proliferation precedes differentiation in IGF-I-stimulated myogenesis. *J Cell Biol* **135**: 431–440.
34. McFarland, DC, Pesall, JE and Gilkerson, KK (1993). The influence of growth factors on turkey embryonic myoblasts and satellite cells *in vitro*. *Gen Comp Endocrinol* **89**: 415–424.
35. Sheehan, SM and Allen, RE (1999). Skeletal muscle satellite cell proliferation in response to members of the fibroblast growth factor family and hepatocyte growth factor. *J Cell Physiol* **181**: 499–506.
36. Tatsumi, R Anderson, JE, Nevoret, CJ, Halevy, O and Allen, RE (1998). HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. *Dev Biol* **194**: 114–128.
37. Beauchamp, JR, Heslop, L, Yu, DS, Tajbakhsh, S, Kelly, RG, Wernig, A *et al.* (2000). Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *J Cell Biol* **151**: 1221–1234.
38. Chambers, RL and McDermott, JC (1996). Molecular basis of skeletal muscle regeneration. *Can J Appl Physiol* **21**: 155–184.
39. Bischoff, R (1994). The satellite cell and muscle regeneration. In Engel, AG and Franzini-Armstrong, C (eds). *Myology: Basic and Clinical.* McGraw-Hill, New York. pp. 97–118.
40. Shen, W, Li, Y, Tang, Y, Cummins, J and Huard, J (2005). NS-398, a cyclooxygenase-2-specific inhibitor, delays skeletal muscle healing by decreasing regeneration and promoting fibrosis. *Am J Pathol* **167**: 1105–1117.
41. Jarvinen, TA, Jarvinen, TL, Kaariainen, M, Kalimo, H and Jarvinen, M (2005). Muscle injuries: biology and treatment. *Am J Sports Med* **33**: 745–764.
42. Branton, MH and Kopp, JB (1999). TGF-β and fibrosis. *Microbes Infect* **1**: 1349–1365.
43. Franklin, TJ (1997). Therapeutic approaches to organ fibrosis. *Int J Biochem Cell Biol* **29**: 79–89.
44. Zhao, J, Sime, PJ, Bringas, P, Gauldie, J and Warburton, D (1999). Adenovirus-mediated decorin gene transfer prevents TGF-beta-induced inhibition of lung morphogenesis. *Am J Physiol* **277**: L412–L422.
45. Gong, R, Rifai, A, Tolbert, EM, Centracchio, JN and Dworkin, LD (2003). Hepatocyte growth factor modulates matrix metalloproteinases and plasminogen activator/plasmin proteolytic pathways in progressive renal interstitial fibrosis. *J Am Soc Nephrol* **14**: 3047–3060.
46. Taniyama, Y, Morishita, R, Nakagami, H, Moriguchi, A, Sakonjo, H, Shokei, K *et al.* (2000). Potential contribution of a novel antifibrotic factor, hepatocyte growth factor, to prevention of myocardial fibrosis by angiotensin II blockade in cardiomyopathic hamsters. *Circulation* **102**: 246–252.
47. Yang, J, Dai, C and Liu, Y (2002). Hepatocyte growth factor gene therapy and angiotensin II blockade synergistically attenuate renal interstitial fibrosis in mice. *J Am Soc Nephrol* **13**: 2464–2477.
48. Norrbom, J, Sundberg, CJ, Ameln, H, Kraus, WE, Jansson, E and Gustafsson, T (2004). PGC-1alpha mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle. *J Appl Physiol* **96**: 189–194.
49. Xiao, X, Li, J and Samulski, RJ (1998). Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* **72**: 2224–2232.

Appendix 3

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 35, pp. 25852–25863, August 31, 2007

Relationships between Transforming Growth Factor-β1, Myostatin, and Decorin

*IMPLICATIONS FOR SKELETAL MUSCLE FIBROSIS**

Received for publication, May 21, 2007 Published, JBC Papers in Press, June 27, 2007, DOI 10.1074/jbc.M704146200

Jinhong Zhu[‡§1], Yong Li[‡¶‖1], Wei Shen[‡§], Chunping Qiao[¶], Fabrisia Ambrosio[‡], Mitra Lavasani[‡§], Masahiro Nozaki[‡¶], Maria F. Branca[‡], and Johnny Huard[‡§¶2]**

*From the [‡]Stem Cell Research Center, Children's Hospital of Pittsburgh, Rangos Research Center, Pittsburgh, Pennsylvania 15213-2583, the [§]Department of Bioengineering, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, the [¶]Department of Orthopaedic Surgery, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213, the [‖]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, and the **Department of Physical Medicine and Rehabilitation, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213-200*

Recent studies have shown that myostatin, first identified as a negative regulator of skeletal muscle growth, may also be involved in the formation of fibrosis within skeletal muscle. In this study, we further explored the potential role of myostatin in skeletal muscle fibrosis, as well as its interaction with both transforming growth factor-β1 and decorin. We discovered that myostatin stimulated fibroblast proliferation *in vitro* and induced its differentiation into myofibroblasts. We further found that transforming growth factor-β1 stimulated myostatin expression, and conversely, myostatin stimulated transforming growth factor-β1 secretion in C2C12 myoblasts. Decorin, a small leucine-rich proteoglycan, was found to neutralize the effects of myostatin in both fibroblasts and myoblasts. Moreover, decorin up-regulated the expression of follistatin, an antagonist of myostatin. The results of *in vivo* experiments showed that myostatin knock-out mice developed significantly less fibrosis and displayed better skeletal muscle regeneration when compared with wild-type mice at 2 and 4 weeks following gastrocnemius muscle laceration injury. In wild-type mice, we found that transforming growth factor-β1 and myostatin co-localize in myofibers in the early stages of injury. Recombinant myostatin protein stimulated myofibers to express transforming growth factor-β1 in skeletal muscles at early time points following injection. In summary, these findings define a fibrogenic property of myostatin and suggest the existence of co-regulatory relationships between transforming growth factor-β1, myostatin, and decorin.

Skeletal muscle injuries are one of the most common injuries encountered in sports, accounting for 10–55% of all sports related injuries (1–3). Despite their clinical significance, current treatments remain conservative, such as the RICE principle (rest, ice, compression, and elevation) and non-steroidal anti-inflammatory drugs. However, increasing evidence shows that the administration of non-steroidal anti-inflammatory drugs decreases regeneration and increases fibrosis by inhibiting inflammation (4–8). Although injured skeletal muscle can spontaneously undergo regeneration, muscle regeneration must compete with the ensuing formation of fibrosis, especially in acute injuries (9–11). The resulting excessive fibrotic tissue might form a dense mechanical barrier that prevents the regenerating muscle fibers from maturing (12, 13), thereby resulting in incomplete skeletal muscle healing (14, 15). Researchers have widely accepted that transforming growth factor-β1 (TGF-β1)[3] is a potent stimulator of fibrosis in various tissues (16–19) and is closely associated with skeletal muscle fibrosis as well (20). TGF-β1 levels are elevated in both dystrophic muscles and injured muscles (21, 22). Researchers have also shown that TGF-β1 effectively induces myofibroblastic differentiation of fibroblasts both *in vitro* and *in vivo* (23, 24). The resulting overgrowth of myofibroblasts is responsible for the ensuing excessive accumulation of fibrotic tissue (23, 24). We have previously reported that TGF-β1 plays a significant role in both the initiation of fibrosis and the induction of myofibroblastic differentiation of myogenic cells in injured skeletal muscle (20, 25). Additionally, we have shown that antifibrosis therapies, such as interferon-gamma (INF-γ), suramin, relaxin, and decorin (DCN), improve the healing of injured muscle both histologically and physiologically by blocking the activity of TGF-β1 (26–32). However, it is unclear whether TGF-β1 acts alone or requires interaction with other molecules during the development of muscle fibrosis. Indeed, recent studies have shown that

* This work was supported by National Institutes of Health Grant AR47973, the Department of Defense Grant W81XWH-06-1-04-06), the Henry J. Mankin and Jean W. Donaldson endowed Chairs, and the Hirtzel Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Stem Cell Research Center, Children's Hospital of Pittsburgh, 4100 Rangos Research Center, 3460 Fifth Ave., Pittsburgh, PA 15213-2583. Tel.: 412-692-7801; Fax: 412-692-7095; E-mail: jhuard@pitt.edu.

[3] The abbreviations used are: TGF-β1, transforming growth factor-β1; MSTN, myostatin; INF, interferon; DCN, decorin; MSTN$^{-/-}$, myostatin knockout; MSTN$^{-/-}$/*mdx* mice, *mdx* mice with myostatin gene knockout; GM, gastrocnemius muscle; PM, proliferation medium; DM, differentiation medium; HS, horse serum; PP1 cells, a population of preplated cells; α-SMA, α-smooth muscle actin; FN, fibronectin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; WT, wide-type; M.O.M., Mouse on Mouse; ECM, extracellular matrix; MRF, muscle regulatory factor; LTP, long-term proliferating; FLST, follistatin; Q-RT-PCR, quantitative reverse transcription-PCR; TβRII, TGF-β1 receptor type II.

myostatin (MSTN), a member of the TGF-β superfamily, may also be involved in fibrosis formation within skeletal muscle (33), although a direct link between MSTN and fibrosis has yet to be identified.

MSTN was initially identified as a negative regulator of muscle development (34), but unlike the ubiquitous expression of TGF-β1, MSTN is predominately expressed in skeletal muscle. MSTN knock-out ($MSTN^{-/-}$) mice, as well as cattle and humans with a naturally occurring MSTN gene mutation, are characterized by a dramatic and widespread increase in skeletal muscle mass (34–36). Interestingly, recent reports suggest that *mdx* mice (an animal model for Duchenne muscular dystrophy) in which expression of the MSTN gene has been ablated ($MSTN^{-/-}$/*mdx*) not only showed better skeletal muscle regeneration but also exhibited decreased fibrosis when compared with *mdx* mice ($MSTN^{+/+}$/*mdx*) (33). These results strongly suggest that MSTN plays an important role in muscle fibrosis. To investigate this possibility, we evaluated the effect of MSTN on fibrosis formation in injured skeletal muscle. Because TGF-β1 plays a major role in the formation of fibrosis, we hypothesized that a relationship between TGF-β1 and MSTN exists. Because DCN has been shown to strongly inhibit fibrosis formation in various tissues via blocking of TGF-β1 activity (26, 27, 37–40), we investigated the potential for DCN to inhibit the activity of MSTN as it does for TGF-β1. Our findings demonstrated that MSTN is involved with fibrosis formation and interacts with TGF-β1 and that DCN has the ability to counteract the action of MSTN. These results contribute to a better understanding of the mechanism of skeletal muscle healing and indicate that MSTN represents a potential pharmacological target for anti-fibrogenic therapy.

EXPERIMENTAL PROCEDURES

Isolation of Fibroblasts from Skeletal Muscle—The preplate technique was used to isolate fibroblasts from skeletal muscle (41). Collagen-coated flasks were used in the isolation process, because fibroblasts adhere more readily to collagen than myoblasts. After 6-week-old female C57BL/6J mice were sacrificed, their gastrocnemius muscles (GMs) were removed and minced into a coarse slurry. The muscle slurry was digested with 0.2% collagenase (type XI) for 1 h, followed by a dispase digestion (grade II, 240 ml) for 30 min, followed by a 0.1% trypsin digestion for a final 30 min at 37 °C. The extracted muscle cells were resuspended in proliferation medium (PM) consisting of Dulbecco's modified Eagle's medium (Invitrogen), 10% horse serum (HS, Invitrogen), 10% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 0.5% chicken embryo extract (Accurate Chemical & Scientific Corp., Westbury, NY) and plated onto collagen-coated flasks. A population of preplated cells (PP1), consisting of mostly fibroblasts that attached within the first 2 h, was collected and used, in these experiments, as skeletal muscle-derived fibroblasts. This preplate technique was also used to isolate long-term proliferating (LTP) cells (muscle-derived stem cell-like cells) from WT and $MSTN^{-/-}$ muscle (41). Two hours after the initial plating, most of the rapidly adhering fibroblasts attached; the remaining nonadherent cells were transferred to a new collagen-coated flask every 24 h. As this process was repeated, the subsequent populations of late-adhering cells were identified as PP2, PP3, PP4, and PP5 in sequence. Following the collection of PP5, the rest of the cell suspension was incubated for an additional 72 h to allow the cells to attach in another collagen-coated flask. The final adherent cells are LTP cells (41).

Cell Culture—The NIH3T3 fibroblast cell line and the C2C12 myoblast cell line were purchased from the American Type Culture Collection (Manassas, VA). The cell lines or isolated PP1 fibroblasts were maintained in PM consisting of Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and 1% penicillin/streptomycin until further needed. PP1 fibroblasts were plated onto collagen-coated 96-well plates for cell-proliferation analysis and onto 6-well plates for the evaluation of α-smooth muscle actin (α-SMA), fibronectin (FN), collagen (types Iα1, IIα2, and IIIα1), and MSTN expression. Following an overnight incubation, PM was replaced with serum-free medium supplemented with a serum replacement (Sigma) consisting of heat-treated bovine serum albumin, heat-treated bovine transferrin, and bovine insulin. This serum replacement does not contain growth factors, steroid hormones, glucocorticoids, or cell adhesion factors. We further supplemented this media with varying concentrations of recombinant human MSTN (Leinco Technologies, Inc., St. Louis, MO) for proliferation assays (0, 100, 500, or 1000 ng/ml) and for Western blot analysis (0, 100, or 500 ng/ml). After incubation for 48 h, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay kit (Roche Diagnostics, Germany) was used to measure cell proliferation ($n = 6$) following the instructions from the manufacturer. Western blot analysis was used to examine α-SMA, FN, and MSTN expression. Some of the above procedures were repeated using NIH3T3 fibroblasts to confirm the effect of MSTN on fibroblasts.

C2C12 myoblasts, a widely used myogenic cell line (42–44), were used to examine whether DCN neutralized the inhibitory effect of MSTN on cell differentiation. We seeded C2C12 myoblasts in 12-well plates in PM at a density of 10,000 cells/well. Following an overnight incubation, PM was replaced with fresh differentiation medium (DM) containing Dulbecco's modified Eagle's medium, 2% HS, and 1% penicillin/streptomycin. We maintained a total of four sets of cultured cells. The control set received only DM, whereas the other sets received DCN alone or 1 μg/ml MSTN combined with 0–50 μg/ml DCN ($n = 3$). Cells were cultured for 5 more days during which DM, MSTN, and DCN were changed every other day. Following a similar procedure, we examined whether recombinant follistatin (FLST) protein stimulated myogenic differentiation of C2C12 myoblasts ($n = 3$), and whether soluble TGF-β1 receptor type II (TβRII, 100 and 1000 ng/ml, R&D Systems, Inc., Minneapolis, MN) was able to attenuate MSTN-inhibited myoblast differentiation ($n = 3$).

Western Blot Analysis—After culturing, the cells were lysed with T-PER® Tissue Protein Extraction Reagent with the addition of protease inhibitors (Pierce). Equal amounts of cellular protein were loaded into each well and separated by 10% SDS-PAGE. Nitrocellulose membrane blotting was performed under standard conditions. The following primary antibodies were used for immunoblotting: mouse anti-β-actin IgG (1:8000, Sigma), mouse anti-glyceraldehyde-3-phosphate dehydrogen-

TABLE 1
Sequence of primer set

Gene name (GenBank™ no.)	Primer pair (S: sense primer, A: anti-sense primer)	PCR products
		bp
Procollagen type Iα1 (BC050014)	S: 5′-GAAGAACTGGACTGTCCCAAC-3′ A: 5′-CCTCGACTCCTACATCTTCTG-3′	103
Procollagen type Iα2 (AK075707)	S: 5′-TCTGGTAAAGAAGGCCCTGTG-3′ A: 5′-GTCCAGGGAATCCGATGTTG-3′	106
Procollagen type IIIα1 (AK041115)	S: 5′-AGGCTGAAGGAAACAGCAAA-3′ (45) A: 5′-TAGTCTCATTGCCTTGCGTG-3′	116
TGF-β1 (BC 013738)	S: 5′-CTAATGGTGGACCGCAACAAC-3′ A: 5′-CACTGCTTCCCGAATGTCTGA-3′	99
18 S rRNA (?)[a]		N/A

[a] Sequences of the primer pairs for 18 S rRNA not provided by Applied Biosystems Inc. for proprietary reasons.

ase IgG (1:5000, Abcam Inc., Cambridge, MA), rabbit anti-MSTN IgG (1:3000, Chemicon, Temecula, CA), mouse anti-α-SMA IgG (1:1000, Sigma), mouse anti-FN IgG (1:3000), and rat anti-TGF-β1 IgG (1:1000, BD Pharmingen, San Jose, CA).

Quantitative RT-RCR—Quantitative RT-PCR (Q-RT-PCR) was used to examine the mRNA expression levels of procollagen (types Iα1, Iα2, and IIIα1) in PP1 fibroblasts treated with MSTN (100, 200, and 500 ng/ml) for 12, 24, and 48 h. The mRNA was extracted using an RNeasy Plus kit (Qiagen). The cDNA templates for Q-RT-PCR were synthesized using a RETROscript® kit (Ambion Inc., Austin, TX). Q-RT-PCR was carried out in an ABI Prism 7000 sequence detector (Applied Biosystems Inc., Foster City, CA) with SYBR Green PCR Master Mix Reagent (Applied Biosystems) as a detector. All target gene expressions were normalized to 18 S rRNA levels. The primer pair of procollagen IIIα1 was from a previous study (45). The primer pairs are displayed in Table 1.

ELISA—Enzyme-linked immunosorbent assay (ELISA) was performed to determine whether recombinant MSTN protein stimulated TGF-β1 secretion in C2C12 myoblasts. C2C12 myoblasts were plated into a 48-well plate and exposed to a range of MSTN concentrations from 0 to 500 ng/ml. Fresh, recombinant MSTN protein was added every 2 days. Cell supernatants were collected at 2 and 4 days ($n = 5$). These supernatants were centrifuged to remove cell debris and stored at −80 °C until the ELISA was performed. The mouse/rat/porcine TGF-β1 immunoassay kit (R&D Systems, Inc.) was used to quantitatively measure the secreted TGF-β1 levels in cell culture supernatants, according to the manufacturer's protocol.

Immunocytochemistry—To monitor the differentiation capacity of the myogenic cells, they were fixed in cold methanol for 2 min after induction of differentiation in 12-well plates. Following a phosphate-buffered saline (PBS) wash, the cells were blocked with 10% HS (Vector Laboratories, Inc., Burlingame, CA) for 30 min, and then incubated with an anti-myosin heavy chain antibody (Sigma) in 2% HS overnight. A negative control was performed by omitting the primary antibody. The next day, after several PBS rinses, the cells were incubated with the secondary antibody goat anti-mouse IgG conjugated with Cy3 (Sigma) for 1 h. Hoechst 33258 dye was used in each experiment to stain cell nuclei. Fusion index (ratio of nuclei in myotubes to all nuclei) was calculated (%) to evaluate myogenic differentiation.

Animal Model—All experimental animal protocols were approved by the Animal Research and Care Committee at Children's Hospital of Pittsburgh (protocols 15-3 and 17-05). C57BL/6 wild-type (WT) (Jackson Laboratories, Bar Harbor, ME) and $MSTN^{-/-}$ mice (7–8 weeks of age) were used in this study. All $MSTN^{-/-}$ mice used were offspring of $MSTN^{-/-}$ homozygotes, and PCR was used to confirm the genotype of all $MSTN^{-/-}$ mice. The RT-PCR test was randomly used to confirm the lack of MSTN gene transcription in $MSTN^{-/-}$ mice throughout the experiments. The skeletal muscle mass of $MSTN^{-/-}$ mice and WT mice were also compared to confirm the desired phenotype. The mice were anesthetized with isoflurane controlled under an IMPAC6 anesthetic delivery machine (VetEquip, Pleasanton, CA). Both GMs of each mouse were laterally lacerated to create an injury model as previously described (27–29). A surgical blade (no. 11) was used to make a lateral laceration through 50% of the muscle width and 100% of the muscle thickness in the area of the GM with the largest diameter. We harvested the mouse GMs at 2 and 4 weeks post-surgery. There were 6–8 mice (12–16 GMs) in the WT and $MSTN^{-/-}$ mouse groups for both time points. The muscles were isolated, removed, and snap-frozen in 2-methylbutane pre-cooled in liquid nitrogen. After Masson's trichrome staining (IMEB Inc., Chicago, IL), Northern Eclipse software (Empix Imaging, Inc., Cheektawaga, NY) was used to measure areas of fibrotic tissue in the injured sites. In each sample, three representative non-adjacent sections were chosen. The ratio of the fibrotic area to the cross-sectional area was used to estimate the extent of fibrosis formation. To determine the skeletal muscle's regeneration efficiency, minor axis diameters (the smallest diameter) of regenerating muscle fibers were measured using Northern Eclipse software on cross-sections of GMs. The diameters of over 350 consecutively centro-nucleated myofibers were measured in each GM.

To analyze the expression of MSTN in the injured GM, 18 8-week-old female C57BL/6 WT mice underwent bilateral GM laceration. Mice were sacrificed at 1, 3, 5, 7, 10, 14, 21, and 30 days after injury ($n = 3$ for each time point), and GMs were harvested, frozen, and stored at −80 °C.

300,000 LTP cells obtained from $MSTN^{-/-}$ mice were transplanted in the GMs of 3 8-week-old *mdx*/scid mice using a protocol previously described (41). The same amount of cells obtained from WT mice was injected into contralateral GMs of *mdx*/scid mice to serve as our control. Mice were sacrificed after 4 weeks, and GMs were frozen in liquid nitrogen. Immunostaining with anti-mouse dystrophin antibody (Abcam Inc.) was performed to detect dystrophin-positive myofibers that regenerated from transplanted cells.

To examine whether the injection of MSTN induced TGF-β1 expression, we injected MSTN (1000 ng in 10 μl of PBS) into the non-injured GM of WT mice. Contralateral GMs were injected with 10 μl of PBS and served as a control. Three WT mice were used at each time point. Mice injected with MSTN were sacrificed at 4, 10, 24, and 48 h after injection ($n = 3$ for each time point). Immunohistochemical staining was performed to detect MSTN and TGF-β1 expression in muscle fibers.

Immunohistochemistry—Frozen GMs were sectioned at 10-μm thickness, and immunohistochemical analysis was performed to detect MSTN and TGF-β1 expression. Tissue sections were fixed in 4% formalin for 5 min followed by two 10-min washes with PBS. The sections were then blocked with 10% HS for 1 h. The rabbit MSTN primary antibody was diluted 1:100 in 2% HS and incubated with sections overnight at 4 °C. The following day, the sections were washed three times with PBS and then incubated with the secondary antibody, goat anti-rabbit IgG conjugated with Cy3 (Sigma). The Mouse-on-Mouse immunodetection kit (M.O.M., Vector Laboratories, Inc.) was then used to stain for TGF-β1 following the manufacturer's protocol. The slides were incubated with M.O.M. blocking reagent for 1 h, washed with PBS, and then incubated with M.O.M. diluent for 5 min. TGF-β1-specific primary antibodies (Vector Laboratories, Inc.) were diluted 1:150 in the M.O.M. diluent and incubated with the slides for 30 min. After washing with PBS, the sections were incubated with anti-mouse IgG conjugated with fluorescein isothiocyanate (diluted 1:200 with M.O.M. diluent, Sigma) for 1 h. Hoechst 33258 dye was used to stain the nuclei. In a separate experiment following a similar procedure, polyclonal rabbit anti-DCN IgG (LF-113, National Institute of Dental Research, Bethesda, MD) was used to stain tissue sections of WT and $MSTN^{-/-}$ GMs 2 weeks after laceration.

Statistical Analysis—All of the results from this study are expressed as the mean ± S.D. The differences between means were considered statistically significant if $p < 0.05$. The Student's *t* test was used to compare the difference in skeletal muscle regeneration, fibrosis formation between $MSTN^{-/-}$ and WT mice, and the myogenic differentiation capacity between $MSTN^{-/-}$ and WT LTP cells. All other data were analyzed by analysis of variance followed by post hoc Tukey's multiple comparison test. *Error bars* on the figures represent the ±S.D. (*, $p < 0.05$; **, $p < 0.01$).

RESULTS

Effects of MSTN on Fibroblasts—MTT proliferation tests showed that, after 48 h of incubation, MSTN significantly stimulated the proliferation of PP1 and NIH3T3 fibroblasts in a dose-dependent manner (Fig. 1*A*). α-SMA, the actin isoform originally found in contractile vascular smooth muscle cells, has been the most reliable marker of myofibroblasts to date (24). Western blot analysis indicated that MSTN (100 and 200 ng/ml) increased α-SMA expression in PP1 and NIH3T3 fibroblasts (Fig. 1*B*). Q-RT-PCR revealed that MSTN stimulated procollagen (type Iα1, Iα2, and IIIα1) mRNA expression at 48 h (Fig. 1*C*). Additionally, MSTN stimulated the expression of FN protein, a component of the extracellular matrix (ECM), in PP1 fibroblasts (Fig. 1*D*).

MSTN Expression in Injured Skeletal Muscle—After laceration injury, different time points were selected to detect MSTN expression in GMs. The degenerative and repair remodeling phases were represented by post-injury time points of 1–3 and 5–30 days following injury, respectively. Immunostaining for MSTN indicated MSTN expression within degenerative myofibers at 1 and 3 days after the injury (data not shown). On day 5, by the time a majority of newly regenerating myofibers was seen, faint MSTN signals were detected in the cytoplasm of regenerating centro-nucleated myofibers (*red fluorescence* and *white arrowheads*), whereas green collagen IV immunostaining indicates basal lamina of myofibers (Fig. 2*A*). MSTN expression was also observed in the nuclei of both the mononuclear cells (*white arrows*) and the regenerating centro-nucleated myofibers (Fig. 2*A*), which is especially obvious in the enlarged image (*white arrowhead* Fig. 2*A*, *inset*). On day 7 (Fig. 2*B*), a decrease in MSTN expression within most of the regenerating myofiber cytoplasm was seen (*white arrowheads*), whereas some myotubes without intact basal lamina were strongly stained with MSTN antibody, which is increased 14 days post-injury (*white arrows*, Fig. 2*C*). The nuclei of myofibers remained MSTN-positive (*yellow arrowhead*, Fig. 2, *B* and *C*, *insets*). MSTN staining disappeared from most regenerated myofibers 30 days after laceration (*white arrowheads*, Fig. 2*D*). Fig. 2, *E*, *F*, *G*, and *H*, depict negative controls of injured muscle at 5, 7, 14, and 30 days after laceration, respectively, where the MSTN antibody was replaced by the non-immune rabbit IgG. Collagen type IV was also stained on these samples to visualize the basal lamina.

Reduced Fibrosis and Enhanced Skeletal Muscle Regeneration in $MSTN^{-/-}$ Mice after Laceration—At 2 weeks following injury, we observed extensive deposition of collagenous tissue in the WT and $MSTN^{-/-}$ mice (data not shown). After 4 weeks, the deepest area of the injured site was filled with regenerating myofibers of large diameter, and the fibrotic region was limited to the superficial zone of the laceration site (Fig. 3*A*). We observed fewer fibrotic connective tissue deposits between regenerating myofibers in the injured muscle of $MSTN^{-/-}$ mice compared with the prominent scar region in the injured WT mouse muscle (Fig. 3*A*). Quantification of fibrotic tissue (*i.e.* the ratio of the fibrotic area to the cross-sectional area) revealed that there was a significantly smaller fibrous area in $MSTN^{-/-}$ skeletal muscle as compared with WT skeletal muscle at 2 weeks (11.5 ± 3.5% *versus* 15.3 ± 3.1%; $p < 0.01$) and at 4 weeks (2.1 ± 0.4 *versus* 6.3 ± 2.1; $p < 0.01$) after injury (Fig. 3*B*).

We used the minor axis diameter (smallest diameter) of centro-nucleated regenerating myofibers to evaluate skeletal muscle regeneration after laceration injury. At 2 weeks after GM laceration, regenerating myofibers were relatively small (data not shown). At 4 weeks, some large, mature myofibers could be observed among the small, centro-nucleated, regenerating myofibers (Fig. 3*C*). Quantification showed that $MSTN^{-/-}$-regenerating myofibers had diameters 38.8% larger than WT myofibers (36.1 ± 2.5 μm *versus* 26.0 ± 2.2 μm, $p < 0.01$) at 2 weeks after laceration, and the mean diameter of regenerating myofibers in $MSTN^{-/-}$ mice remained 21.1% larger than the

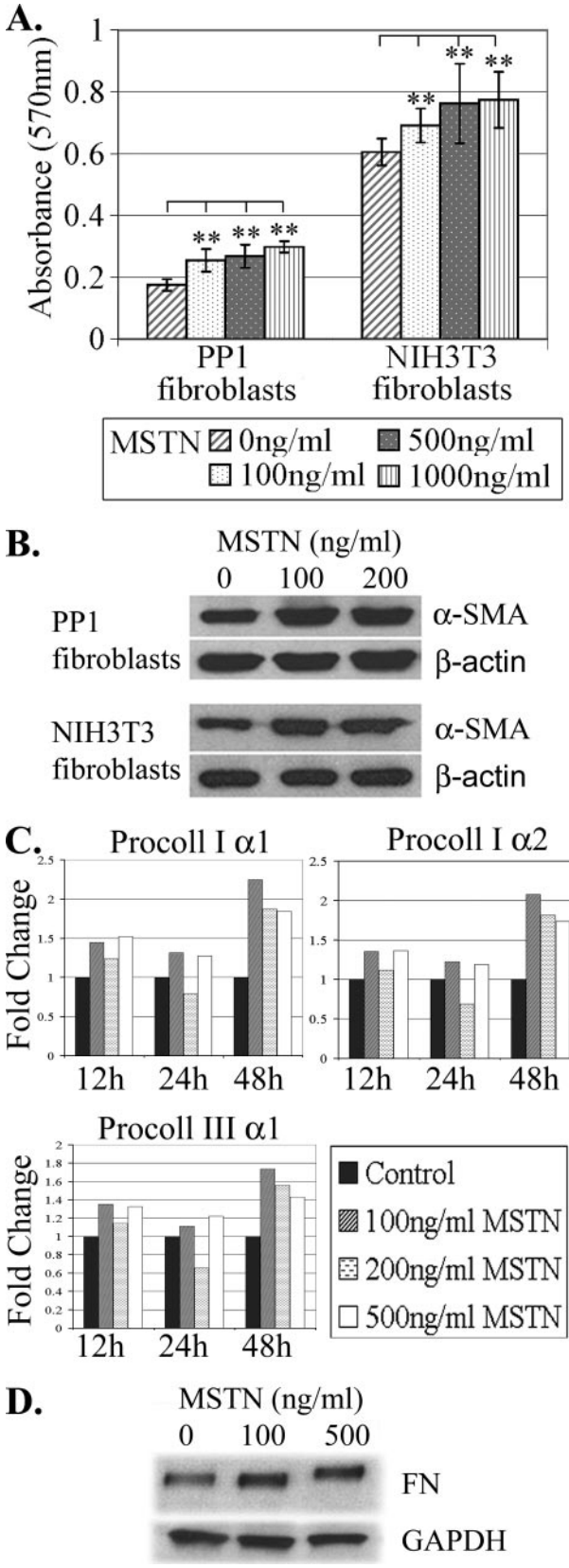


FIGURE 1. **MSTN stimulated fibroblast proliferation and fibrotic protein expression in fibroblasts.** *A*, both muscle-derived fibroblasts (*PP1*) and NIH3T3 fibroblasts were cultured with MSTN, varying in concentration from 0 to 1000 ng/ml for 48 h. Cell proliferation was determined by MTT assay. These results are presented as absorbance values ($n = 6$) of purple formazan crystal at 570 nm, which directly correlates to the number of living cells. Fibroblasts were cultured in DM for 2 days with the addition of various concentrations of MSTN. Expressions of different proteins were analyzed by Western blot. *B*, the expression of α-SMA in PP1 fibroblasts or NIH3T3 fibroblast is shown. *C*, Q-RT-PCR analysis of procollagen (types Iα1, Iα2, and IIIα1) mRNA expression in PP1 fibroblasts treated with MSTN. Results are presented as the ratio against the gene expression in the control. *D*, expression of FN in PP1 fibroblasts after MSTN treatment (*, $p < 0.05$; **, $p < 0.01$).

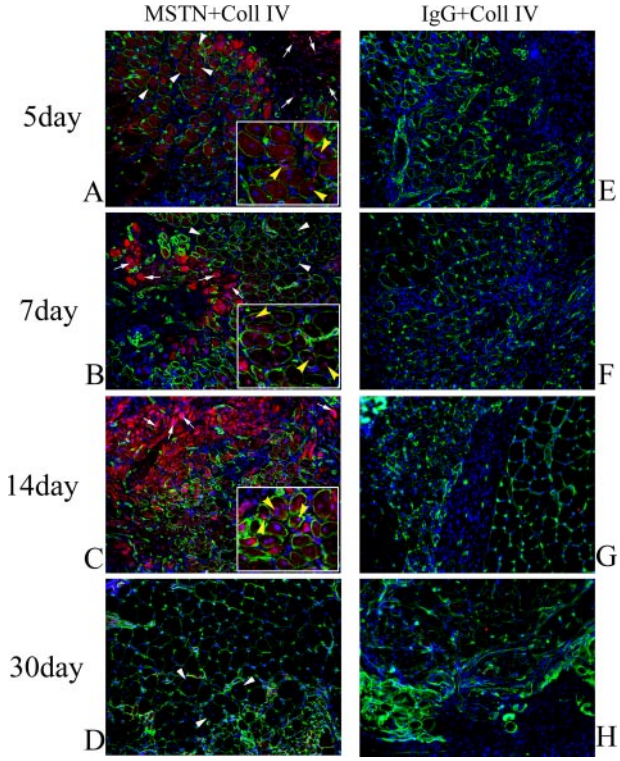


FIGURE 2. **MSTN localization in injured GMs.** *A–H*, GMs from WT mice were harvested at different time points after laceration injury, and frozen sections were immunostained with rabbit anti-MSTN and goat anti-collagen type IV antibodies. MSTN, collagen IV, and cell nuclei are *red*, *green*, and *blue*, respectively (*A–D*). Non-immune rabbit IgG was used as negative control for rabbit anti-MSTN antibody (*E–H*), but were stained with the collagen type IV antibody. *A*, at 5 days, faint MSTN signals could be detected in the cytoplasm of newly formed myofibers (*white arrowheads*) with basal lamina, and a relatively higher MSTN staining can be observed in the nuclei of regenerating myofiber (*yellow arrowhead*) and mononuclear cells (*white arrow*). *B*, at 7 days, MSTN staining is not evident in the cytoplasm of most regenerating myofibers (*white arrowhead*), whereas some of the regenerating small myotubes without basal lamina show intense MSTN staining in the cytoplasm (*white arrows*). *Yellow arrowheads* in the *inset* indicate positive signal in nuclei of regenerating myofibers. *C*, at 14 days, there were more MSTN-positive myotubes without basal lamina (*white arrow*). *Yellow arrowheads* in the *inset* indicate positive signal in nuclei of regenerating myofibers. *D*, at 30 days, most of regenerating myofibers were MSTN-negative (*arrowheads*). (Magnification, ×200; *inset* magnification, ×400.)

mean diameter of regenerating myofibers in the WT mice (37.7 ± 2.7 μm *versus* 31.1 ± 1.8 μm, $p < 0.01$) 4 weeks after injury (Fig. 3*D*). The distribution of the regenerating myofiber diameters showed that there was an increase in the percentage of larger regenerating myofibers in MSTN$^{-/-}$ mice compared with WT mice (*e.g.* ~7.38% of regenerating myofiber diameters in MSTN$^{-/-}$ mice fell into a range of 50–55 μm *versus* 1.92% of those in WT mice).

Improved Myogenic Potential with MSTN$^{-/-}$ LTP Cells—LTP cells were isolated from WT and MSTN$^{-/-}$ mice. When we cultivated these MSTN$^{-/-}$ LTP cells in low serum medium, they differentiated into myotubes that were significantly larger

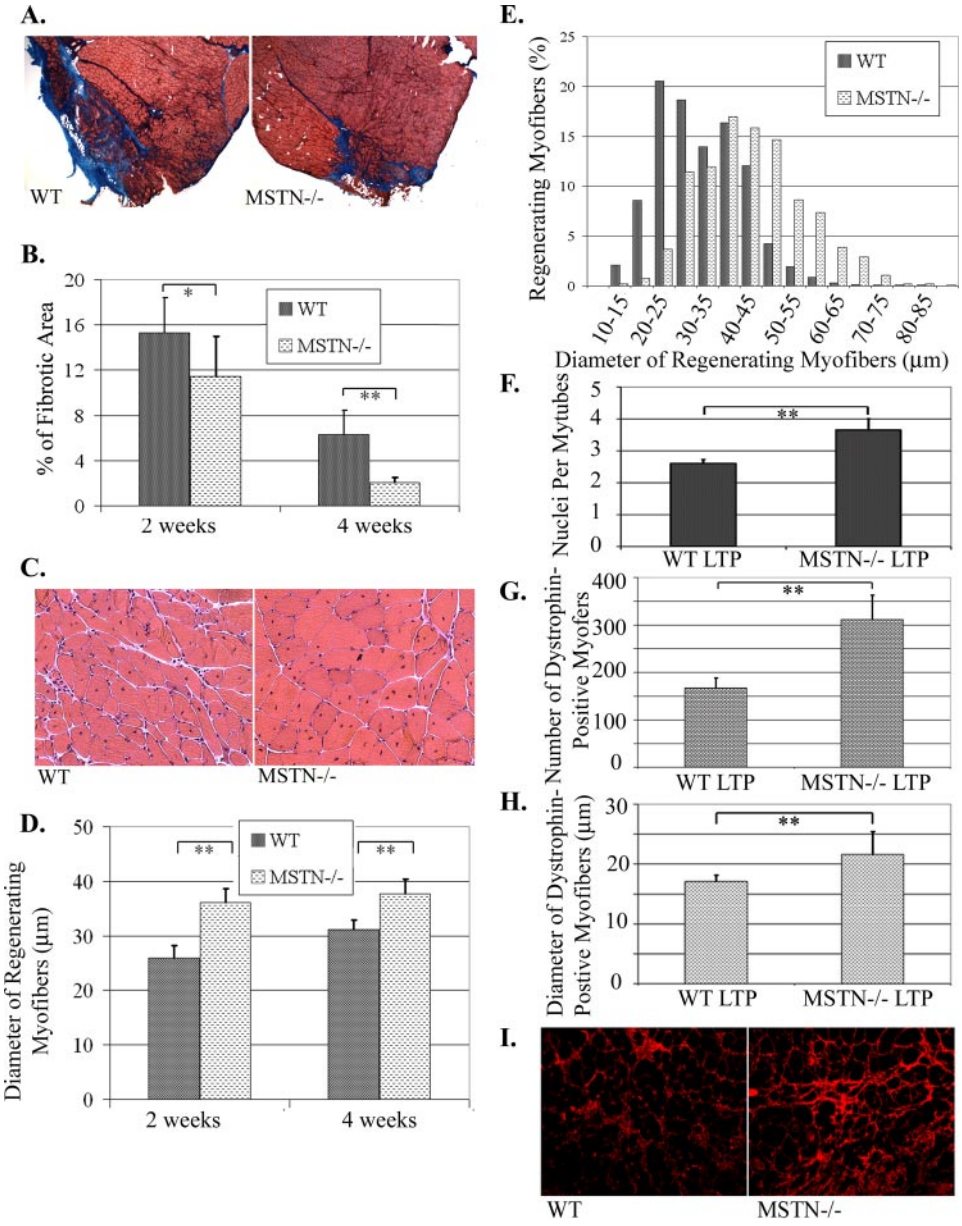


FIGURE 3. **Inhibition of MSTN favors skeletal muscle regeneration.** *A*, sections from injured WT and MSTN$^{-/-}$ GMs were stained with Masson's trichrome-staining protocol 4 weeks after laceration to determine fibrotic tissue levels. As a result, collagenous tissue is stained *blue*. *B*, quantification of fibrotic tissue of WT *versus* MSTN$^{-/-}$ GMs 2 and 4 weeks after laceration. *C*, myofibers in WT and MSTN$^{-/-}$ GMs were visualized by hematoxylin and eosin staining 4 weeks after laceration. Regenerating myofibers were distinguished by their centralized nuclei. *D*, quantification of the diameters of regenerating myofibers. *E*, the distribution of regenerating myofiber diameters at 4 weeks after laceration injury. *F*, myogenic differentiation capacity of WT and MSTN$^{-/-}$ LTP cell *in vitro*. *In vivo*, transplantation of MSTN$^{-/-}$ LTP into *mdx*/scid mice led to a high number of dystrophin-positive muscle fibers when compared with WT LTP. *G*, the number of dystrophin-positive myofibers was counted. *H*, the diameter of dystrophin-positive myofibers was measured. *I*, increased DCN immunostaining in injured skeletal muscle of MSTN$^{-/-}$ mice compared with WT mice 2 weeks after laceration. DCN (*red*) is detected in the ECM between myofibers. (Magnifications: in *C* and *I*, ×200; in *A*, ×100; *, $p < 0.05$; **, $p < 0.01$.)

(more nuclei per myotube, $n = 3$) than the myotubes formed by the fusion of WT LTP cells (Fig. 3*F*). When we injected the MSTN$^{-/-}$ LTP cells into the muscle of *mdx*/scid mice, they regenerated significantly more dystrophin-positive muscle fibers than did the WT LTP (Fig. 3*G*). These regenerating muscle fibers were also significantly larger in diameter (Fig. 3*H*).

Elevated DCN Expression in Injured MSTN$^{-/-}$ Mice—To investigate the underlying mechanism for improved muscle healing in MSTN$^{-/-}$ mice, we examined the expression of DCN, a molecule that has been shown to decrease fibrosis and enhance muscle regeneration (20, 27) in injured MSTN$^{-/-}$ skeletal muscle. Immunohistochemical staining revealed that there was more abundant DCN expression in the regenerating skeletal muscle of MSTN$^{-/-}$ mice than that of WT mice 2 weeks after injury (Fig. 3*I*). This higher level of DCN expression may be involved with the increased regeneration and decreased fibrosis observed in the injured muscle of MSTN$^{-/-}$ mice.

Relationship between TGF-β1 and MSTN$^{-/-}$—Western blot analysis showed that the levels of MSTN in C2C12 myoblasts treated with different concentrations of TGF-β1 were elevated in a dose-dependent manner when compared with non-treated controls, suggesting that TGF-β1 stimulates MSTN expression in C2C12 myoblasts (Fig. 4*A*). After incubation with increasing concentrations of recombinant MSTN protein, MSTN was shown to stimulate TGF-β1 expression in C2C12 myoblasts (especially with the highest dose) at 4 days post-stimulation (Fig. 4*B*). Furthermore, ELISA showed that MSTN significantly increased TGF-β1 secretion by C2C12 myoblasts in a dose-dependent manner at 2 and 4 days. After 4 days of stimulation with MSTN (500 ng/ml), C2C12 myoblasts secreted ~2-fold more TGF-β1 as compared with control cells (Fig. 4*C*). Q-RT-PCR revealed that MSTN (100, 200, and 500 ng/ml) also increased TGF-β1 mRNA expression 48 h post-stimulation (Fig. 4*D*).

PP1 fibroblasts did not express detectable MSTN protein. However, after treatment with MSTN (100 and 200 ng/ml) for 48 h, PP1 fibroblasts began to express MSTN as indicated by Western blot analysis (Fig. 4*E*). MSTN also stimulated MSTN expression in C2C12 myoblasts (Fig. 4*E*). MSTN-induced MSTN autocrine expression in PP1 fibroblasts is reduced by soluble TβRII, which blocks the TGF-β1 signaling pathway (Fig. 4*F*). Moreover, our results indicated that soluble TβRII was also able to restore MSTN-inhibited C2C12 myoblast differentiation (Fig. 4*G*). We also examined whether exogenous

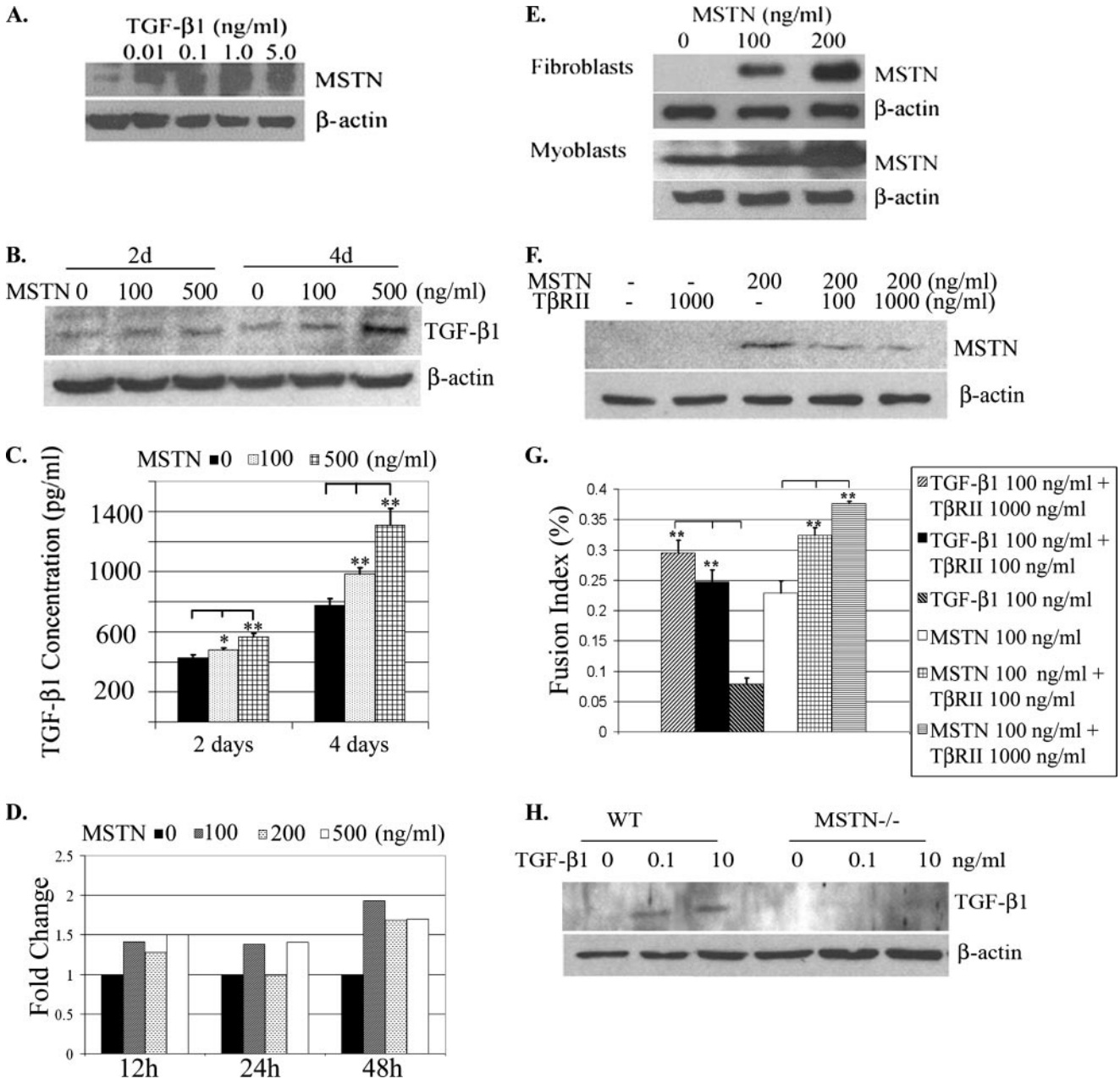


FIGURE 4. **The relationship between TGF-β1 and MSTN *in vitro*.** *A*, Western blot analysis of MSTN expression in C2C12 myoblasts treated with different concentrations of TGF-β1 ranging from 0 to 5.0 ng/ml for 48 h. *B*, C2C12 myoblasts were treated with different concentrations of MSTN in DM. Cell lysates were collected at 2 and 4 days to examine TGF-β1 expression by Western blot; *C*, while the conditioned medium was collected at the same time points, the levels of TGF-β1 in the medium were also analyzed by ELISA. *D*, Q-RT-PCR for TGF-β1 after MSTN treatment (100, 200, and 500 ng/ml) in PP1 fibroblasts. *E*, the level of MSTN expression in PP1 fibroblasts *and C2C12 myoblasts* treated with MSTN recombinant protein. *F*, Western blots were used to determine MSTN expression level in PP1 fibroblasts after cells were treated with either MSTN or both MSTN and soluble TβRII for 48 h. *G*, C2C12 myoblasts were cultured in DM with different treatments, TGF-β1, MSTN, TGF-β1 and TβRII, or MSTN and TβRII, for 4 days. Fusion indexes were used to access impacts of treatments on C2C12 myoblast differentiation. *H*, myoblasts isolated from WT, and MSTN$^{-/-}$ GMs were grown for 48 h under stimulation by TGF-β1. Western blot analysis was used to detect TGF-β1 expression in WT and MSTN $^{-/-}$ cells (*, $p < 0.05$; **, $p < 0.01$).

TGF-β1 recombinant protein was able to stimulate autocrine expression of TGF-β1 in MSTN$^{-/-}$ muscle cells as it does in C2C12 myoblasts (20). We observed that exogenous TGF-β1 could induce its autocrine expression in WT primary myoblasts but not on primary MSTN$^{-/-}$ myoblasts (Fig. 4*H*).

In vivo, We observed co-expression of TGF-β1 (*green*) and MSTN (*red*) in degenerative myofibers 1 and 3 days after laceration injury (*white arrow*, Fig. 5*A*). By day 5, MSTN was detected mainly in the nuclei of the regenerating myofibers (*white arrowhead*) with the exception of a few MSTN-positive necrotic myofibers, whereas TGF-β1 was present in the surrounding ECM (*white arrow*). MSTN was still detected in the nuclei of regenerating myofibers 21 days after injury (*white arrow*, Fig. 5*A*). The injection of MSTN into non-injured GMs induced TGF-β1 expression in the myofibers at 4, 10, and 24 h after injection. As shown in Fig. 5*B*, MSTN (*red*) and TGF-β1 (*green*) were co-expressed in myofibers at

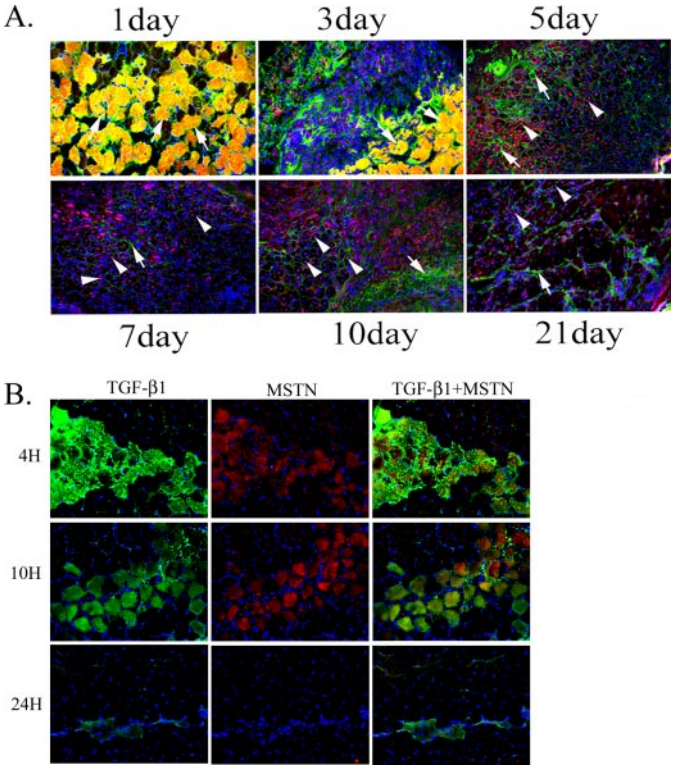


FIGURE 5. **The relationship between TGF-β1 and MSTN *in vivo*.** *A*, both GMs of each adult WT mouse underwent laceration injury. Mice GMs were harvested at the indicated times. Double staining of TGF-β1 (*green*) and MSTN (*red*) was performed. In the 1- and 3-day images, a *white arrow* indicates degenerative myofibers. At all other time points, the *white arrow* and *arrowhead* indicate ECM and nuclei of myofibers, respectively. *B*, co-localization of TGF-β1 and MSTN in myofibers after recombinant MSTN protein injection. We injected 1000 ng of MSTN protein in 10 μl of PBS into GMs of WT mice. Mice were sacrificed at different time points after injection. Frozen sections of GMs were double-stained with anti-TGF-β1 and anti-MSTN antibodies (magnification, ×200).

4 and 10 h. After 24 h, MSTN disappeared, and only a few TGF-β1-positive myofibers could be observed.

DCN Counteracts the Effect of MSTN—As previously shown in Fig. 1*A*, 0.1 μg/ml MSTN significantly stimulated PP1 fibroblast proliferation. This dosage was selected to examine whether DCN could reduce the proliferative influence of MSTN on PP1 fibroblasts. After PP1 fibroblasts were incubated with MSTN and exposed to varying concentrations of DCN for 48 h, MTT assay revealed that the addition of DCN significantly repressed the stimulatory effect of MSTN on PP1 proliferation in a dose-dependent manner as expected (Fig. 6*A*). These findings are comparable to a previous report showing that DCN blocked the stimulatory effect of TGF-β1 on PP1 fibroblasts (27).

Our earlier results indicated that MSTN induced its own expression, in an autocrine manner, in PP1 fibroblasts (Fig. 4*E*). Therefore, we examined the ability of DCN to block the MSTN autocrine expression in PP1 fibroblasts. As previously shown, PP1 fibroblasts that were not treated with MSTN failed to express detectable MSTN protein, whereas PP1 fibroblasts treated with MSTN showed a high level of MSTN expression in comparison to the control (Figs. 4*E* and 6*B*). However, DCN decreased MSTN autocrine expression by PP1 fibroblasts in a dose-dependent manner (Fig. 6*B*).

Our previous experiments showed that 1 μg/ml MSTN almost completely inhibited myoblast differentiation (data not shown). Therefore, we chose this dose to assess whether DCN treatment could reverse MSTN-inhibited myogenic differentiation in C2C12 cells. Except for the control cells, the cultures were treated with DCN alone or 1 μg/ml MSTN combined with increasing concentrations of DCN (0–50 μg/ml). Following a 5-day incubation, DCN-treated groups (data not shown) and controls showed widespread myosin heavy chain-positive myotubes, whereas cells treated with MSTN alone contained only a few myotubes (Fig. 6*C*). The addition of DCN reversed the inhibition of MSTN on myogenic differentiation, as indicated by the increase in the number and size of myotubes in comparison to the MSTN-treated group (Fig. 6*C*). Measurements showed that DCN treatment promoted C2C12 myoblast differentiation by significantly increasing fusion indexes in a dose-dependent manner (Fig. 6*D*), suggesting that DCN attenuated the inhibitory effect of MSTN and, thereby, stimulated myoblast fusion.

Inhibitory Effects of DCN on MSN May Be Mediated by FLST—To further explore whether DCN regulated MSTN activity via an intermediate molecule, we investigated the effect of DCN on the expression of FLST, which is able to bind to MSTN and suppress its activity (46). We found an up-regulation of FLST expression by C2C12 myoblasts 48 and 72 h after addition of 10 μg/ml DCN (Fig. 7*A*). Our results also revealed the ability of FLST to stimulate myogenic differentiation, which was demonstrated by the presence of larger myotubes containing more nuclei in comparison to the control group (Fig. 7*B*). In a dose-dependent manner, FLST treatment led to a significant increase in fusion index (Fig. 7*C*) compared with the control group, suggesting that FLST promotes myogenic differentiation and accelerates the maturation of myotubes.

DISCUSSION

MSTN has been drawing more and more attention due to mounting evidence indicating that inhibition of MSTN significantly improves skeletal muscle diseases such as muscle dystrophy. But, the role of MSTN in injured skeletal muscle and its relationships with other molecules such as TGF-β1 and DCN (important key factors in muscle healing) remain unknown. Recent studies reported by Yamanouchi *et al.* (47) highlight the expression of MSTN in fibroblasts in injured skeletal muscle, suggesting that fibroblasts may be a source of MSTN. Previously, we have shown that TGF-β1 significantly promotes proliferation of PP1 fibroblasts (27). Here, our *in vitro* study shows that MSTN activates fibroblasts by stimulating fibroblast proliferation and inducing their expression of α-SMA analogous to that of TGF-β1. Like TGF-β1 (48), MSTN may transiently attract fibroblasts into an injury site, further inducing them to express MSTN in an autocrine fashion; they then differentiate into myofibroblasts, thereby accelerating the deposition of the ECM. Researchers widely believe that prolonged presence and excessive activity of myofibroblasts is associated with the abnormal accumulation of ECM components in injured and diseased tissue (49, 50). Moreover, MSTN has been shown to induce procollagen (types Iα1, Iα2, and IIIα1), mRNA, and FN protein expression in PP1 fibroblasts. McCroskery *et al.* (51) recently confirmed the correlation of MSTN expression to the

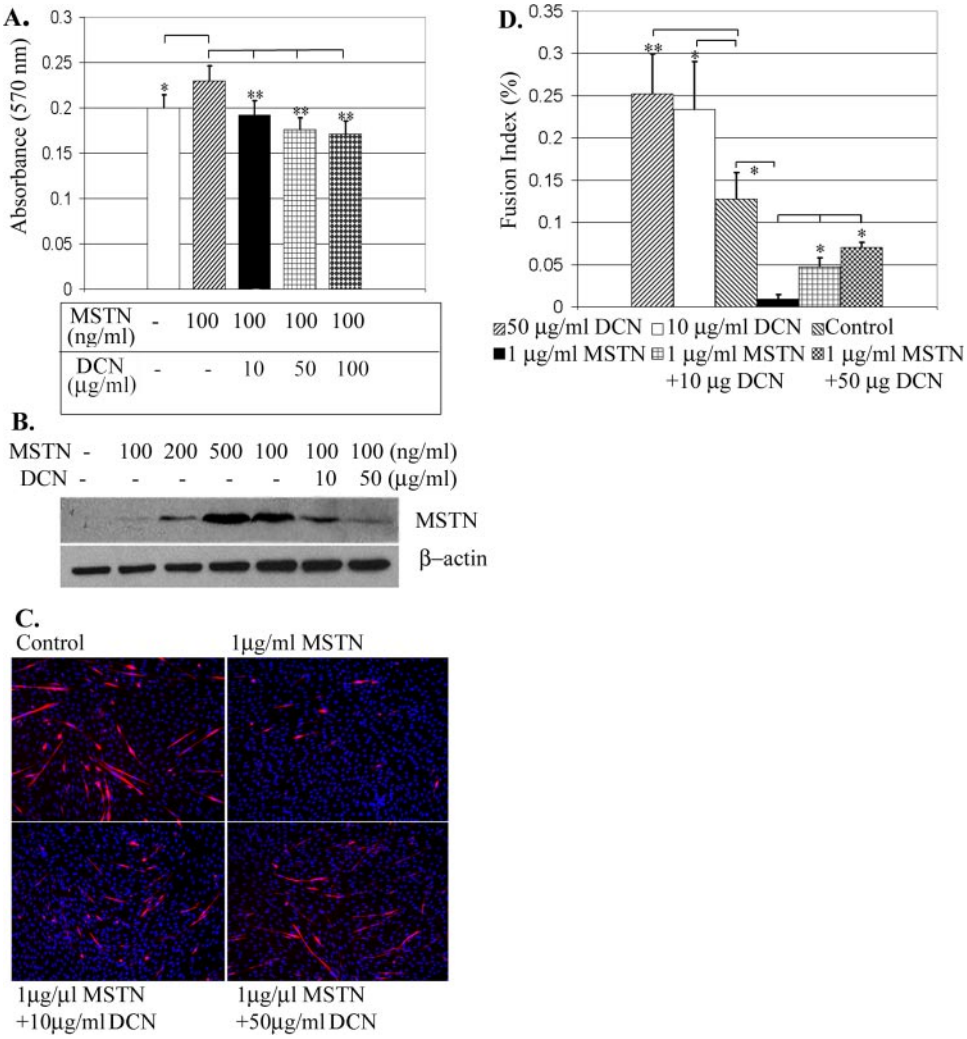


FIGURE 6. **DCN blocks the effects of MSTN on PP1 fibroblasts and C2C12 myoblasts.** *A*, PP1 fibroblasts were treated for 48 h with 100 ng/ml MSTN or combinations of MSTN and DCN. Non-treated cell cultures were used as a control. MTT assay was performed to assess cell proliferation. *B*, after incubation of PP1 fibroblasts with MSTN, or a combination of MSTN and DCN, Western blot analysis was performed to determine whether DCN reduced the autocrine expression of MSTN in PP1 fibroblasts stimulated with MSTN. *C*, C2C12 myoblasts were cultured without treatment, with 1 μg/ml MSTN alone, or co-incubated with 1 μg/ml MSTN and different concentrations of DCN for 5 days. Myotubes were monitored by anti-skeletal myosin heavy chain immunostaining; nuclei were stained by Hoechst 33258 (magnification, ×100). *D*, fusion indexes were determined to estimate the differentiation capacity of C2C12 myoblasts in response to different treatments.

formation of fibrosis by showing less fibrosis formation in the notexin-damaged tibialis anterior muscle in MSTN$^{-/-}$ mice 4 weeks after injury as compared with WT mice. Given the results collected in our *in vitro* study, we hypothesized that a lack of MSTN in knock-out mice would decrease the proliferation of fibroblasts and reduce their production of collagenous tissue in injured skeletal muscle. This was made evident by a significant decrease in the formation of fibrosis in MSTN$^{-/-}$ mice at 2 and 4 weeks after injury when compared with WT mice. Moreover, we found an elevated expression level of DCN, an inhibitor of TGF-β1, in injured MSTN$^{-/-}$ skeletal muscles compared with injured WT muscles at 2 weeks after injury. In accordance with this result, increased DCN mRNA has been observed in regenerating MSTN$^{-/-}$ muscle (51). Increased DCN might inhibit the effect of TGF-β1, thereby partially explaining the reduced fibrosis and enhanced regeneration in injured MSTN$^{-/-}$ muscle. To understand the mechanism by which MSTN$^{-/-}$ muscle displays less fibrosis than WT muscle after injury, the expression levels of TGF-β1 in injured WT mice *versus* that expressed in injured MSTN$^{-/-}$ mice should be compared more closely.

As members of the TGF-β superfamily, TGF-β1 and MSTN share many similarities in structure, signaling pathway, and function (52, 53). It has also been shown that TGF-β1 plays a critical role in skeletal muscle fibrosis after injury (20, 26–32). Because both TGF-β1 and MSTN promote fibrosis, it is very important to understand the potential relationships between these two molecules. Recent reports demonstrated that exogenous TGF-β1 strongly stimulated the expression of MSTN in C2C12 myoblasts (44). In fact, our *in vitro* data show that TGF-β1 increases MSTN expression in C2C12 myoblasts (and *vice versa*), and TGF-β1 and MSTN are found to co-localize in the same myofibers shortly after MSTN injection or after injury.

We found that MSTN is able to induce its autocrine expression in both fibroblasts and myoblasts. In the presence of soluble TβRII, MSTN autocrine expression in fibroblasts is decreased. We have known that MSTN inhibits C2C12 myoblast differentiation. When TβRIIs are blocked by soluble TβRII, the ability of MSTN to inhibit C2C12 myoblast differentiation is reduced. Apart from that, Q-RT-PCR results show that MSTN also stimulates TGF-β1 mRNA expression in PP1 fibroblasts. Our previous study has shown that TGF-β1 is able to induce autocrine expression of TGF-β1 in C2C12 myoblasts (20), nevertheless, our present data revealed that TGF-β1 failed to induce its autocrine expression in MSTN$^{-/-}$ primary muscle cells. Although TGF-β1 and MSTN may target different cell membrane receptors (52), our results suggest that they may also bind to the same receptor, indicating that their signaling may be somehow related. It is likely, then, that the inducement of skeletal muscle fibrosis by TGF-β1 is partially mediated by its interaction with MSTN. However, the mechanism by which TGF-β1 interacts with MSTN to cause fibrosis warrants further investigation.

Satellite cells serve as a reservoir of myogenic progenitor cells for the repair and maintenance of skeletal muscle. MSTN negatively regulates self-renewal and differentiation of satellite

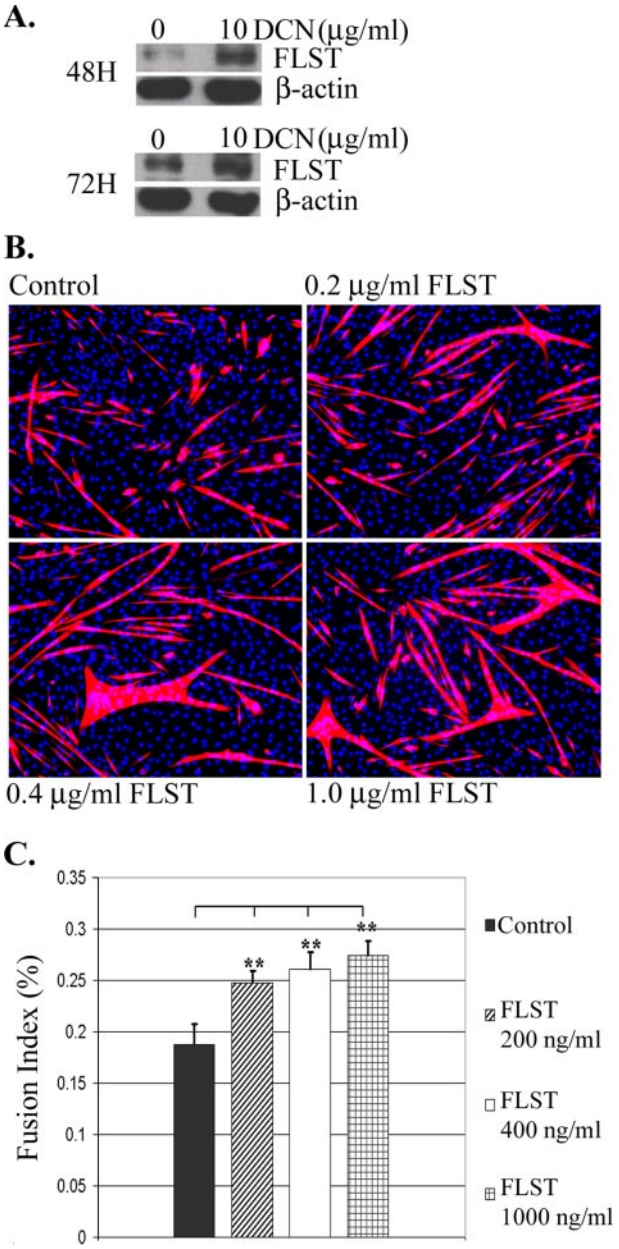


FIGURE 7. **The elevated FLST expression by DCN and the capacity of FLST to enhance myogenic differentiation of C2C12 myoblasts.** *A*, DCN increased the expression of FLST in C2C12 myoblasts 48 and 72 h after treatment. *B*, immunofluorescence analysis of myotubes. C2C12 myoblasts were maintained in DM for 5 days in the presence of different concentrations of FLST. Myotubes were double-labeled with an antibody recognizing skeletal myosin heavy chain and with the fluorescent nuclear dye Hoechst 33258 (magnification, ×100). *C*, fusion indexes were calculated to evaluate the degree of C2C12 myoblast differentiation upon FLST stimulation.

cells (54) and decreases the expression of members of the basic helix-loop-helix muscle regulatory factors (MRF) (MyoD, Myf5, mrf4, and myogenin) (43, 55). MSTN$^{-/-}$ mice show an increased number of satellite cells activated and differentiated toward a myogenic lineage (54). In this study, our data demonstrate that MSTN$^{-/-}$ mice contain regenerating myofibers with significantly larger diameters than WT mice at 2 and 4 weeks after GM laceration. The increased number of satellite cells in MSTN$^{-/-}$ mice could, in part, explain the enhanced regeneration revealed by the larger diameter of regenerated myofibers in MSTN$^{-/-}$ mice compared with WT mice. Indeed, it has been reported that blocking MSTN signals by isolating myoblasts from transgenic mice carrying the mutated MSTN receptor results in improved success of myoblast transplantation in *mdx* mice compared with normal myoblasts (56). Our results show that MSTN$^{-/-}$ LTP more readily undergo myogenic differentiation *in vitro* and regenerate skeletal muscle *in vivo* in a more effective manner than wild-type cells.

Furthermore, high levels of MSTN protein have been reported within necrotic fibers in the skeletal muscles of rats damaged by notexin (57), and Western blot analysis revealed the up-regulation of MSTN protein at early time points following notexin-induced injury in rat skeletal muscle (58). Interestingly, it has been shown that MSTN interferes with the chemotaxis of macrophages *in vitro* (51); recombinant MSTN protein significantly reduces the migration of macrophages and myoblasts toward chemoattractants *in vitro*, which likely promotes skeletal muscle regeneration (51). These results suggest that MSTN could impede recruitment of macrophages and myoblasts into the injured site *in vivo*. Macrophages infiltrate damaged tissue to remove debris that could hinder muscle regeneration. Macrophages also secrete a variety of growth factors and cytokines that have chemotactic and/or mitogenic effects on muscle precursor cells, thereby accelerating muscle regeneration (59–63). Compared with WT mice, MSTN$^{-/-}$ mice have shown elevated recruitment of macrophages and myoblasts and an accelerated inflammatory response after muscle injury (51). These results suggest that the earlier initiation of skeletal muscle regeneration in the injured skeletal muscle of MSTN$^{-/-}$ mice compared with the injured muscle of WT mice may be due, in part, to accelerated removal of muscle debris. When we monitored the expression of MSTN at the injured site for up to 30 days after injury, we observed an intense expression of MSTN in the cytoplasm of degenerative myofibers 1 and 3 days after laceration. On day 5 after injury, MSTN signal was detected in the cytoplasm of regenerating myofibers. Our results show that the MSTN signal decreases with maturation of regenerating myofibers. Interestingly, there is strong MSTN immunostaining in regenerating small myotubes lacking basal lamina 7 and 14 days post-injury. During skeletal muscle healing (following active muscle regeneration at early time points after injury) fibrosis initiates ~1 week post-injury, and peaks at 4 weeks (10, 15, 64). Li *et al.* (20) reported that some regenerating myofibers probably differentiate into myofibroblasts to contribute the formation of fibrosis. This correlation between fibrosis development and increased MSTN and TGF-β1 expression (20) in the early phase of healing may suggest the differentiation of regenerating myotubes/myofibers into myofibroblasts and a potential interaction between TGF-β1, MSTN, and DCN, as previously hypothesized (65).

MSTN-positive signals were also seen within the nuclei of the newly formed fibers at 5, 7, and 10 days post-injury. The nuclear localization of MSTN is supported by previous studies indicating that MSTN was detected in the nuclei of myoblasts and myotubes (66). Consequently, MSTN protein might modulate the muscle fiber regeneration process through the early events of phagocytosis and inflammation (57) and later control myofiber maturation. In this way, MSTN seems to act as a reg-

ulatory molecule that is produced by the tissue to specifically suppress and control the size of muscle growth and development (67).

DCN, a small chondroitin-dermatan sulfate leucine-rich proteoglycan, exists ubiquitously in the ECM. Due to its binding to and inhibition of TGF-β1, DCN has been used as a potent anti-fibrosis agent in various organs and tissues (26, 27, 37–40), including skeletal muscle (26, 27). However, the ability of DCN to regulate MSTN activity is still unknown. DCN, which is composed of a core protein and a single glycosaminoglycan chain (68, 69), has the ability to bind to TGF-β1 due to the fact that the core protein of DCN contains two binding sites for TGF-β1 (70). Similarly, Miura *et al.* (65) have shown that DCN, or the core protein of DCN, directly binds to active MSTN molecules to block MSTN-mediated inhibition of C2C12 myoblast proliferation. The actual location of the MSTN binding site in the DCN core protein and evidence that shows whether TGF-β1 and MSTN competitively bind to DCN are topics for further investigation. Of further interest is the possibility that DCN may regulate MSTN by influencing another intermediate molecule like FLST, an antagonist of MSTN (46). Our results not only show that DCN reduces the effects of MSTN on fibroblasts and myoblasts, but also indicates that it stimulates the expression of FLST in C2C12 myoblasts. Exogenous FLST then stimulates C2C12 myoblast differentiation, which is probably due to FLST's neutralization of endogenous MSTN. These results indicate that the effect of DCN on MSTN may be related to the up-regulation of FLST, which would consequently suppress MSTN activity. Nevertheless, more experiments that would, for example, examine the effect of DCN on FLST knock-out cells, need to be done to establish the role of FLST in DCN-inhibited MSTN activity. Furthermore, we have shown that TGF-β1 probably plays a role in the MSTN signaling pathway, because TGF-β1-soluble receptor antagonizes, at least in part, the effect of myostatin on muscle cells. Overall, DCN probably regulates MSTN activity via three ways: (i) directly binding MSTN, (ii) indirectly down-regulate MSTN by binding to TGF-β1, and (iii) indirectly down-regulating MSTN by stimulating FLST expression.

In summary, our results suggest the following: (i) MSTN stimulates the formation of fibrosis in skeletal muscle after injury, (ii) TGF-β1 and MSTN up-regulate the expression level of each other, and (iii) DCN is capable of inhibiting MSTN activity as it does for TGF-β1. These results, combined with the fact that TGF-β1 plays a key role in skeletal muscle fibrosis and that DCN reduces fibrosis in injured skeletal muscle, suggest that TGF-β1 and MSTN probably act together; they synergistically amplify the fibrotic process in injured or diseased skeletal muscles resulting in greater fibrosis than either could induce individually.

Our findings may help to further increase the understanding of the mechanism by which $MSTN^{-/-}$ mice show decreased fibrosis and enhanced regeneration after injury and suggest that the inhibition of MSTN might be a new therapeutic approach for improving skeletal muscle healing through enhancement of regeneration and reduction of fibrosis.

Acknowledgments—We thank Dr. Se-Jin Lee (Johns Hopkins University) for the $MSTN^{-/-}$ breeder mice; Lynn Bauer for breeding the $MSTN^{-/-}$ mice utilized in this report; Bin Sun for Q-RT-PCR; and David Humiston, Ryan Sauder, and Shannon Bushyeager for their excellent editorial work.

REFERENCES

1. Beiner, J. M., and Jokl, P. (2001) *J. Am. Acad. Orthop. Surg.* **9,** 227–237
2. Garrett, W. E., Jr. (1996) *Am. J. Sports Med.* **24,** Suppl. 6, S2–S8
3. Jarvinen, M. J., and Lehto, M. U. (1993) *Sports Med.* **15,** 78–89
4. McLennan, I. S. (1985) *Exp. Neurol* **89,** 616–621
5. McLennan, I. S. (1987) *Muscle Nerve* **10,** 801–809
6. Mishra, D. K., Friden, J., Schmitz, M. C., and Lieber, R. L. (1995) *J. Bone Joint Surg. Am.* **77,** 1510–1519
7. Obremsky, W. T., Seaber, A. V., Ribbeck, B. M., and Garrett, W. E., Jr. (1994) *Am J. Sports Med.* **22,** 558–561
8. Shen, W., Li, Y., Tang, Y., Cummins, J., and Huard, J. (2005) *Am J. Pathol.* **167,** 1105–1117
9. Schultz, E., Jaryszak, D. L., and Valliere, C. R. (1985) *Muscle Nerve* **8,** 217–222
10. Li, Y., Cummins, H. J., and Huard, J. (2001) *Curr. Opin. Orthop.* **12,** 409–415
11. Huard, J., Li, Y., and Fu, F. H. (2002) *J. Bone Joint Surg. Am.* **84-A,** 822–832
12. Jarvinen, M. (1975) *Acta Pathol. Microbiol. Scand. A* **83,** 269–282
13. Jarvinen, M. (1976) *Acta Chir. Scand.* **142,** 47–56
14. Jarvinen, M., and Sorvari, T. (1975) *Acta Pathol. Microbiol. Scand. A* **83,** 259–265
15. Lehto, M., Jarvinen, M., and Nelimarkka, O. (1986) *Arch. Orthop. Trauma Surg.* **104,** 366–370
16. Border, W. A., and Noble, N. A. (1994) *N. Engl. J. Med.* **331,** 1286–1292
17. Lijnen, P. J., Petrov, V. V., and Fagard, R. H. (2000) *Mol. Genet. Metab.* **71,** 418–435
18. Waltenberger, J., Lundin, L., Oberg, K., Wilander, E., Miyazono, K., Heldin, C. H., and Funa, K. (1993) *Am. J. Pathol.* **142,** 71–78
19. Yamamoto, T., Noble, N. A., Miller, D. E., and Border, W. A. (1994) *Kidney Int.* **45,** 916–927
20. Li, Y., Foster, W., Deasy, B. M., Chan, Y., Prisk, V., Tang, Y., Cummins, J., and Huard, J. (2004) *Am. J. Pathol.* **164,** 1007–1019
21. Bernasconi, P., Torchiana, E., Confalonieri, P., Brugnoni, R., Barresi, R., Mora, M., Cornelio, F., Morandi, L., and Mantegazza, R. (1995) *J. Clin. Invest.* **96,** 1137–1144
22. Gosselin, L. E., Williams, J. E., Deering, M., Brazeau, D., Koury, S., and Martinez, D. A. (2004) *Muscle Nerve* **30,** 645–653
23. Desmouliere, A., Geinoz, A., Gabbiani, F., and Gabbiani, G. (1993) *J. Cell Biol.* **122,** 103–111
24. Tomasek, J. J., Gabbiani, G., Hinz, B., Chaponnier, C., and Brown, R. A. (2002) *Nat. Rev. Mol. Cell. Biol.* **3,** 349–363
25. Li, Y., and Huard, J. (2002) *Am. J. Pathol.* **161,** 895–907
26. Sato, K., Li, Y., Foster, W., Fukushima, K., Badlani, N., Adachi, N., Usas, A., Fu, F. H., and Huard, J. (2003) *Muscle Nerve* **28,** 365–372
27. Fukushima, K., Badlani, N., Usas, A., Riano, F., Fu, F., and Huard, J. (2001) *Am. J. Sports Med.* **29,** 394–402
28. Foster, W., Li, Y., Usas, A., Somogyi, G., and Huard, J. (2003) *J. Orthop. Res.* **21,** 798–804
29. Chan, Y. S., Li, Y., Foster, W., Horaguchi, T., Somogyi, G., Fu, F. H., and Huard, J. (2003) *J. Appl. Physiol.* **95,** 771–780
30. Chan, Y. S., Li, Y., Foster, W., Fu, F. H., and Huard, J. (2005) *Am. J. Sports Med.* **33,** 43–51
31. Li, Y., Negishi, S., Sakamoto, M., Usas, A., and Huard, J. (2005) *Ann. N. Y. Acad. Sci.* **1041,** 395–397
32. Negishi, S., Li, Y., Usas, A., Fu, F. H., and Huard, J. (2005) *Am. J. Sports Med.* **33,** 1816–1824
33. Wagner, K. R., McPherron, A. C., Winik, N., and Lee, S. J. (2002) *Ann. Neurol.* **52,** 832–836
34. McPherron, A. C., Lawler, A. M., and Lee, S. J. (1997) *Nature* **387,** 83–90
35. McPherron, A. C., and Lee, S. J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,**

12457–12461
36. Williams, M. S. (2004) *N. Engl. J. Med.* **351,** 1030–1031; author reply 1030–1031
37. Giri, S. N., Hyde, D. M., Braun, R. K., Gaarde, W., Harper, J. R., and Pierschbacher, M. D. (1997) *Biochem. Pharmacol.* **54,** 1205–1216
38. Grisanti, S., Szurman, P., Warga, M., Kaczmarek, R., Ziemssen, F., Tatar, O., and Bartz-Schmidt, K. U. (2005) *Invest. Ophthalmol. Vis. Sci.* **46,** 191–196
39. Huijun, W., Long, C., Zhigang, Z., Feng, J., and Muyi, G. (2005) *Exp. Mol. Pathol.* **78,** 17–24
40. Shimizu, I. (2001) *Curr. Drug. Targets Infect. Disord.* **1,** 227–240
41. Qu-Petersen, Z., Deasy, B., Jankowski, R., Ikezawa, M., Cummins, J., Pruchnic, R., Mytinger, J., Cao, B., Gates, C., Wernig, A., and Huard, J. (2002) *J. Cell Biol.* **157,** 851–864
42. Thomas, M., Langley, B., Berry, C., Sharma, M., Kirk, S., Bass, J., and Kambadur, R. (2000) *J. Biol. Chem.* **275,** 40235–40243
43. Langley, B., Thomas, M., Bishop, A., Sharma, M., Gilmour, S., and Kambadur, R. (2002) *J. Biol. Chem.* **277,** 49831–49840
44. Budasz-Rwiderska, M., Jank, M., and Motyl, T. (2005) *J. Physiol. Pharmacol.* **56,** Suppl. 3, 195–214
45. Yamazaki, K., Fukata, H., Adachi, T., Tainaka, H., Kohda, M., Yamazaki, M., Kojima, K., Chiba, K., Mori, C., and Komiyama, M. (2005) *Mol. Reprod. Dev.* **72,** 291–298
46. Amthor, H., Nicholas, G., McKinnell, I., Kemp, C. F., Sharma, M., Kambadur, R., and Patel, K. (2004) *Dev. Biol.* **270,** 19–30
47. Yamanouchi, K., Soeta, C., Naito, K., and Tojo, H. (2000) *Biochem. Biophys. Res. Commun.* **270,** 510–516
48. Pierce, G. F., Mustoe, T. A., Lingelbach, J., Masakowski, V. R., Griffin, G. L., Senior, R. M., and Deuel, T. F. (1989) *J. Cell Biol.* **109,** 429–440
49. Phan, S. H. (2002) *Chest* **122,** Suppl. 6, 286S–289S
50. Thannickal, V. J., Toews, G. B., White, E. S., Lynch, J. P., 3rd, and Martinez, F. J. (2004) *Annu Rev. Med.* **55,** 395–417
51. McCroskery, S., Thomas, M., Platt, L., Hennebry, A., Nishimura, T., McLeay, L., Sharma, M., and Kambadur, R. (2005) *J. Cell Sci.* **118,** 3531–3541
52. Rebbapragada, A., Benchabane, H., Wrana, J. L., Celeste, A. J., and Attisano, L. (2003) *Mol. Cell. Biol.* **23,** 7230–7242
53. Zhu, X., Topouzis, S., Liang, L. F., and Stotish, R. L. (2004) *Cytokine* **26,** 262–272
54. McCroskery, S., Thomas, M., Maxwell, L., Sharma, M., and Kambadur, R. (2003) *J. Cell Biol.* **162,** 1135–1147
55. Joulia, D., Bernardi, H., Garandel, V., Rabenoelina, F., Vernus, B., and Cabello, G. (2003) *Exp. Cell Res.* **286,** 263–275
56. Benabdallah, B. F., Bouchentouf, M., and Tremblay, J. P. (2005) *Transplantation* **79,** 1696–1702
57. Kirk, S., Oldham, J., Kambadur, R., Sharma, M., Dobbie, P., and Bass, J. (2000) *J. Cell. Physiol.* **184,** 356–363
58. Mendler, L., Zador, E., Ver Heyen, M., Dux, L., and Wuytack, F. (2000) *J. Muscle Res. Cell Motil.* **21,** 551–563
59. Cantini, M., Massimino, M. L., Bruson, A., Catani, C., Dalla Libera, L., and Carraro, U. (1994) *Biochem. Biophys. Res. Commun.* **202,** 1688–1696
60. Chazaud, B., Sonnet, C., Lafuste, P., Bassez, G., Rimaniol, A. C., Poron, F., Authier, F. J., Dreyfus, P. A., and Gherardi, R. K. (2003) *J. Cell Biol.* **163,** 1133–1143
61. Lescaudron, L., Peltekian, E., Fontaine-Perus, J., Paulin, D., Zampieri, M., Garcia, L., and Parrish, E. (1999) *Neuromuscul. Disord.* **9,** 72–80
62. Merly, F., Lescaudron, L., Rouaud, T., Crossin, F., and Gardahaut, M. F. (1999) *Muscle Nerve* **22,** 724–732
63. Robertson, T. A., Maley, M. A., Grounds, M. D., and Papadimitriou, J. M. (1993) *Exp. Cell Res.* **207,** 321–331
64. Menetrey, J., Kasemkijwattana, C., Fu, F. H., Moreland, M. S., and Huard, J. (1999) *Am. J. Sports Med.* **27,** 222–229
65. Miura, T., Kishioka, Y., Wakamatsu, J., Hattori, A., Hennebry, A., Berry, C. J., Sharma, M., Kambadur, R., and Nishimura, T. (2006) *Biochem. Biophys. Res. Commun.* **340,** 675–680
66. Artaza, N. J., Bhasin, S., Mallidis, C., Taylor, W., Ma, K., and Gonzalez-Cadavid, F. N. (2002) *J. Cell. Physiol.* **190,** 170–179
67. Kocamis, H., and Killefer, J. (2002) *Domest. Anim. Endocrinol.* **23,** 447–454
68. Yamaguchi, Y., and Ruoslahti, E. (1988) *Nature* **336,** 244–246
69. Krusius, T., and Ruoslahti, E. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 7683–7687
70. Schonherr, E., Broszat, M., Brandan, E., Bruckner, P., and Kresse, H. (1998) *Arch. Biochem. Biophys.* **355,** 241–248

DECORIN INTERACTS WITH MYOSTATIN ACTIVITY – IMPLICATIONS FOR SKELETAL MUSCLE HEALING

*Zhu J; *Li Y; +*Huard J
+*Stem Cell Research Center, Children's Hospital of Pittsburgh and Departments of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA
jhuard@pitt.edu

Introduction:

Skeletal muscle injuries are one of the most common injuries occuring in field of sports medicine. Muscle injuries can heal spontaneously via regeneration, but fibrosis usually impedes this process which results in incomplete functional recovery. We have demonstrated that TGF-β1 plays a major role in the initiation of fibrosis in injured muscle [1]. Decorin, a small chondroitin-dermatan sulphate leucine-rich proteoglycan, has been shown to improve skeletal muscle healing histologically and physiologically through a concomitant increase in muscle regeneration and a decrease in fibrosis [2]. It is widely accepted that the antifibrotic effect of decorin primarily results from its capability of directly binding to and neutralizing TGF-β1 [3]. The identification of a new TGF-β family member, myostatin (MSTN) [4], has inspired us to further explore the mechanism by which decorin improves skeletal muscle healing. Our unpublished data showed that 1) MSTN stimulates proliferation and myofibrotic differentiation of fibroblasts in vitro; 2) that the injured skeletal muscle of MSTN knockout (MSTN-/-) mice contains significantly less fibrous scar tissue than observed in the injured muscle of normal wild-type (WT) mice and 3) regenerating myofibers of MSTN-/- mice are significantly larger in diameter than those in the injured muscle of WT mice. Thus MSTN, like TGF-β1, appears to play an important role in fibrosis. We performed this study to investigate whether decorin could inhibit the activity of MSTN as it does with TGF-β1.

Methods:

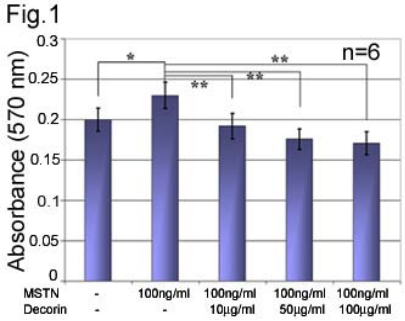
Proliferation assay and Western blot analysis: PP1 fibroblasts were plated onto collagen-coated 96-well plates for cell proliferation analysis. Following overnight attachment, normal medium was replaced with serum free medium plus serum replacement (Sigma, St Louis, MO) in the presence of varying concentrations of decorin and 100ng/ml of recombinant human MSTN. After an additional incubation for 48 h, an MTT cell proliferation assay kit (Roche Diagnostics, Germany) was used to measure cell proliferation (n = 6) following instructions from the manufacturer. Western blot was used to examine β-actin and MSTN expression. To test whether decorin neutralized the inhibitory effect of MSTN on myogenic cell differentiation, C2C12 myoblasts were seeded in 12-well plates at a density of 10,000cells/well. After overnight incubation, medium was replaced with fresh differentiation medium (DM) containing DMEM, 2% HS, and 1% P/S, with or without addition of 1μg/ml MSTN. This was concomitant with the addition of 0-100 μg/ml decorin (n = 3). Cells were cultured for another 5 days. DM, MSTN, and decorin were changed every other day.

Injury model: All experiments in this study were in accordance with research protocols approved by the ARCC of Children's Hospital of Pittsburgh. MSTN-deficient mice (MSTN-/-) and C57BL/6J wild-type mice (control) were used to establish an injury model of skeletal muscle laceration [2]. GMs of both MSTN-/- and wild-type mice were harvested 2 weeks after laceration. Decorin immunostaining was performed to evaluate decorin expression in injured skeletal muscle. Student's *t*-test or one way ANOVA were used to determine significance ($P < 0.05$).

Results:

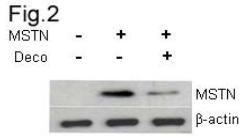
Blockade of MSTN effect on fibroblasts by Decorin:. After PP1 fibroblasts were incubated with MSTN and varying concentrations of decorin for 48 h, MTT assay revealed that 0.1μg/ml MSTN significantly stimulated PP1 fibroblast proliferation whereas the addition of decorin significantly repressed the MSTN stimulatory effect on PP1 proliferation (Fig 1).

Fig.1



Decorin blocks MSTN autocrine expression in fibroblasts: After 48 h incubation, PP1 fibroblasts did not express detectable MSTN protein, and PP1 fibroblasts treated with MSTN showed a high level of MSTN expression. However, MSTN expression in PP1 fibroblasts treated with both MSTN and decorin was reduced to only a detectable level (Fig 2).

Fig.2



Blockade of MSTN effect on C2C12 myoblast differentiation by Decorin

Following a 5-day incubation, controls showed widespread skeletal muscle heavy chain (MHC) positive myotubes whereas cells treated with MSTN alone contained few myotubes (Fig 3). The addition of decorin reversed the inhibition of MSTN on myogenic differentiation (Fig 3). Quantification showed that decorin treatment significantly promoted C2C12 myoblast fusion and increased the number and size of the myotubes in the presence of MSTN (Fig 3) in a dose dependent manner

Elevated decorin expression in injured MSTN-/- mice

Immnunohistochemical staining revealed that there was a significantly higher level of decorin expression in the regenerating skeletal muscle of MSTN-/- mice than that of WT mice 2 weeks after injury (Fig 4).

Fig.3

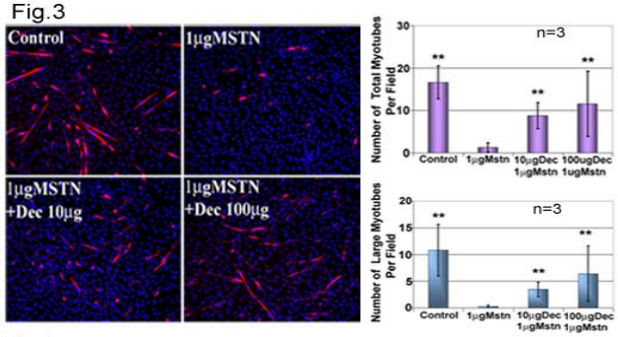
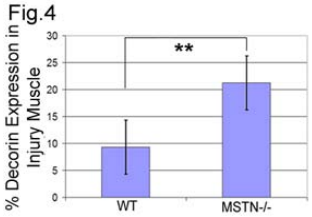


Fig.4



Discussion:

In this study, we showed that decorin blocked the effects of MSTN on both fibroblasts and myoblasts in vitro. Decorin reduced the stimulating effects of MSTN on fibroblasts and the MSTN autocrine expression of fibroblasts. Moreover, decorin rescued the inhibitory effect of MSTN on myoblast differentiation. The ability of decorin to bind to TGF-β1 is due to the fact that core protein of decorin contains two binding sites for TGF-β1. Similarly, it has been recently shown that decorin, or the core protein of decorin, can directly bond to active MSTN molecules to block MSTN-mediated inhibition of C2C12 myoblast proliferation [5]. The location of the MSTN binding site in the decorin core protein and whether TGF-β1 and MSTN competitively bind to decorin are topics for further investigation. These findings provide us a better understanding of how decorin can improve skeletal muscle healing after injury.

Acknowledgments: This work was supported by funding from the Henry J. Mankin Endowed Chair for Orthopaedic Research at the University of Pittsburgh, the William F. and Jean W. Donaldson Chair at Children's Hospital of Pittsburgh, the Hirtzel Foundation, and the National Institutes of Health (R01 AR47973.). We thank Dr. Se-Jin Lee (Johns Hopkins University) for providing us the MSTN-/- mice.

References: 1. Li Y et al. Am J Pathol 2004;164:1007–19. 2. Fukushima et al. Am J Sports Med 2001 ; 29(4) : 394-402. 3.Yamaguchi et al. Nature 1988 ; 336(6196) : 244-6. 4. McPherron AC et al. Nature 1997;387:83–90. 5. Miura et al. Biochem Biophys Res Commun 2006; 340(2): 675-80.

**Department of Bioengineering, University of Pittsburgh

FOLLISTATIN IMPROVES SKELETAL MUSCLE HEALING AFTER INJURY

* **Zhu J; *Li Y; Branca M ; +*Huard. J
*Stem Cell Research Center, Children's Hospital of Pittsburgh and Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA
jhuard@pitt.edu

INTRODUCTION

Skeletal muscle injuries account for 10 to 30% of all sports related injuries. Muscle injuries can heal via spontaneous regeneration; however, as a result of severe injury, incomplete functional recovery can increase the time an athlete is off the field. Decorin has been shown to effectively improve skeletal muscle healing by reducing fibrosis and accelerating skeletal muscle regeneration [1]. Aside from decorin, Follistatin (FLST) has been drawing more attention do to the fact that increasing knowledge about this molecule reveals it as a promising new target for therapeutic improvement of skeletal muscle healing. FLST was initially described as an inhibitor of follicle-stimulating hormone decades ago. More and more evidence revealed that FLST is capable of binding and neutralizing many members of the TGF-β superfamily such as myostatin (MSTN) and activin [2, 3]. Recently, it was found that FLST overexpression mice (FLST OE) showed dramatic increase in skeletal muscle mass compared to wild-type control mice [4]. Activin is implicated in the formation of fibrosis in many tissues and organs such as skin, liver, and kidney [2, 3]. Furthermore, our unpublished data demonstrated that MSTN is a fibrosis stimulator in the skeletal muscle and blocking MSTN significantly reduces the formation of fibrosis in injured skeletal muscle of mice. Taken together, we hypothesize that FLST may improve the healing of injured skeletal muscle. In this study, transgenic FLST OE mice were used to investigate the influence of FLST on skeletal muscle healing.

METHODS

Myoblast differentiation assay: C2C12 myoblasts were plated onto collagen-coated 12-well plates with normal medium overnight. The following day, the medium was replaced with low serum medium (2% horse serum) plus different concentrations of FLST (Sigma, St. Louis, MO). The cells were cultured for 6 additional days. Medium and recombinant FLST protein were changed every other day. Myotubes were monitored by myosin heavy chain (MHC) immunostaining, and the fusion capacity of C2C12 myoblasts was evaluated by determining the number of myonuclei per myotube (n = 3).

Western blot: C2C12 myoblasts were plated onto collagen-coated 6-well plates. Following overnight attachment, normal medium was replaced with low serum medium in the presence of decorin (Sigma, St. Louis, MO). Protein samples were collected at 48 and 72h and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Goat anti-follistatin antibody (Santa Cruz Biotechnology, INC. Santa Cruz, CA) was used to detect FLST expression in C2C12 myoblasts

Animal model: All experiments in this study were approved by the Children's Hospital of Pittsburgh IACUC. C57BL/6J wide-type (WT) and FLST OE mice (7 to 8 weeks of age) were used for all experiments (n = 6). Both gastrocnemius muscles (GMs) of each mouse underwent bilateral laceration [1]. The GMs were harvested 4 weeks after laceration, and Masson's Trichrome staining (nuclei [black], muscle [red], collagen [blue]) was performed to identify fibrous scar tissue in the injured muscles. Northern Eclipse software (Empix Imaging, Inc.) was used to measure areas of fibrous scar tissue and regenerated muscle within the injury site.

Statistics: One-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison test or Student's *t* test was used to determine significance ($P < 0.05$) throughout this study. (*) represents that $P < 0.05$, and (**) represents that $P < 0.01$;

RESULTS

FLST induces large myotube formation in vitro: Our results reveal the ability of FLST to stimulate myogenic differentiation as shown in Fig 1. Myotubes treated with FLST were larger and contained more nuclei than the control (Fig. 1A). FLST treatment leads to a significant increase in the number of nuclei per myotubes in a dose dependent manner (Fig. 1B) compared to control, suggesting that FLST promotes myogenic fusion and accelerates the maturation of myotubes.

Reduced fibrosis in FLST OE mice after GM laceration: The results of Masson's Trichrome histochemistry revealed significantly less fibrous scar tissue in the FLST OE GMs than in the WT GMs (Fig. 2A, B).

Decorin stimulates FLST expression in C2C12 myoblasts: We found that decorin up-regulated FLST expression in C2C12 myoblasts after 48h and 72h of culture (Fig.3).

Fig.1A

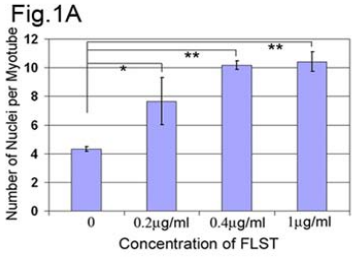


Fig.1B.

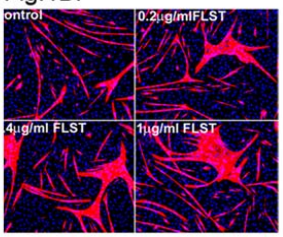


Fig.2A

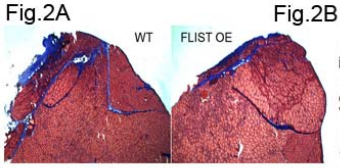


Fig.2B

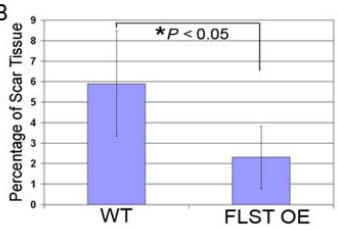
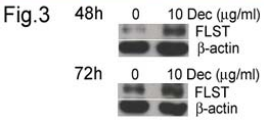


Fig.3



DISCUSSION

We have demonstrated that TGF-β1 plays a significant role in both the initiation of fibrosis and the induction of myofibroblastic differentiation by myogenic cells in injured skeletal muscle [6]. Although, FLST improves skeletal muscle healing, it does not seem to block fibrosis in injured skeletal muscle through inhibition of TGF-β1. Our result showed that FLST failed to block the inhibition that TGF- β1 has on myogenic differentiation (data not shown). Most likely, FLST inhibits the formation of fibrosis by antagonizing the activities of activin and MSTN [2, 3]. FLST overexpression improves skeletal muscle healing by reducing fibrosis. Although FLST significantly increases myoblast differentiation in vitro, FLST OE mice did not show increased diameter of regenerating myofibers compared to WT mice at 4 weeks after GM laceration. This suggests that FLST is involved with an alternative mechanism for skeletal muscle healing. Moreover, we also showed that decorin treatment elevated FLST expression in C2C12 myoblasts. It seems likely that, apart from neutralizing TGF- β1, decorin also reduces fibrosis in injured skeletal muscle by up-regulating the intermediate molecule, FLST. The skeletal muscle healing process is a complex process, in which many molecules interact with each other, and our findings provide insight into one aspect of this network.

ACKNOWLEDGMENTS

This work was supported by funding from the Henry J. Mankin Endowed Chair for Orthopaedic Research at the University of Pittsburgh, the William F. and Jean W. Donaldson Chair at Children's Hospital of Pittsburgh, the Hirtzel Foundation, and the National Institutes of Health (R01 AR47973). The FLST OE mice were a gift from Dr. Se-Jin Lee (Johns Hopkins University).

REFERENCES

1. Fukushima et al. Am J Sports Med 2001;29(4):394–402. 2. Phillips et al. Front Neuroendocrinol 1998 ;225 :127-32. 3. Amthor, H., et al., Dev Biol 2004. 270(1): 19-30. 4. Lee SJ et al. Proc Natl Acad Sci USA 2001;98:9306–11. 5. Sulyok et al. Mol Cell Endocrinol 2004;118:3531–41. 6. Li Y et al. Am J Pathol 2004;164:1007–19.

AFFILIATED INSTITUTIONS FOR CO-AUTHORS

**Department of Bioengineering, University of Pittsburgh

Suramin can enhance the skeletal muscle healing by blocking myostation

*Nozaki M, *Li Y, *Zhu J, *Kenji Uehara, *Ambrosio F, **Fu FH, +*Huard J
*Stem Cell Research Center, Children's Hospital of Pittsburgh and **Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA
jhuard@pitt.edu

INTRODUCTION:

Muscle injuries are very common musculoskeletal problems encountered in sports medicine. Although this type of injury is capable of healing, complete functional recovery is hindered by the development of scar tissue formation triggered by TGF-β1 [1]. We already reported suramin can effectively prevent the formation of fibrotic scar and block the proliferative effect of TGF-β1 on fibroblasts and can stimulate the differentiation on myogenic cells *in vitro* (unpublished data). Thus suramin can enhance muscle regeneration in the lacerated and strain-injured muscle [2, 3]. Furthermore we also reported blockade of myostatin (MSTN), a member of TGF-β super family, by decorin, other anti-fibrotic agent, showed enhancement of the fusion on myoblasts and inhibitory effect of fibroblast proliferation *in vitro* [4]. This finding brought us more interest to investigate another pathway of suramin to regulate fibroblasts and myoblasts by blocking the effect of MSTN. We performed this study to examine whether suramin would block MSTN's proliferative effect on fibroblasts and inhibitory effect of myoblasts differentiation *in vitro* and reduce the expression of MSTN in injured muscle to improve muscle healing *in vivo*, using an animal model of muscle contusion.

MATERIALS AND METHODS:

3T3 cell proliferation assay: 3T3 fibroblasts were cultured in 96 well plates (n=5) with DMEM containing 2% serum replacement (Sigma, St Louis, MO) and different concentration of MSTN (0 and 1 μg/ml) and suramin (0 and 50 μg/ml). Three days after incubation CellTiter Cell Proliferation Assay kit (Promega, Madison, WI) was used to measure cell proliferation.

C2C12 cell differentiation assay: C2C12 myoblasts were cultured in 24 well plates (n=4) with differentiation medium (DMEM containing 2% horse serum and 1% penicillin/streptomycin) containing different concentration of MSTN (0 and 1 μg/ml) and suramin (0, 1,and 25 μg/ml). Three days after incubation immunocytochemistry of myosin heavy chain was done and the fusion index was assessed by counting the number of nuclei in differentiated myotubes as a percentage of the total number of nuclei.

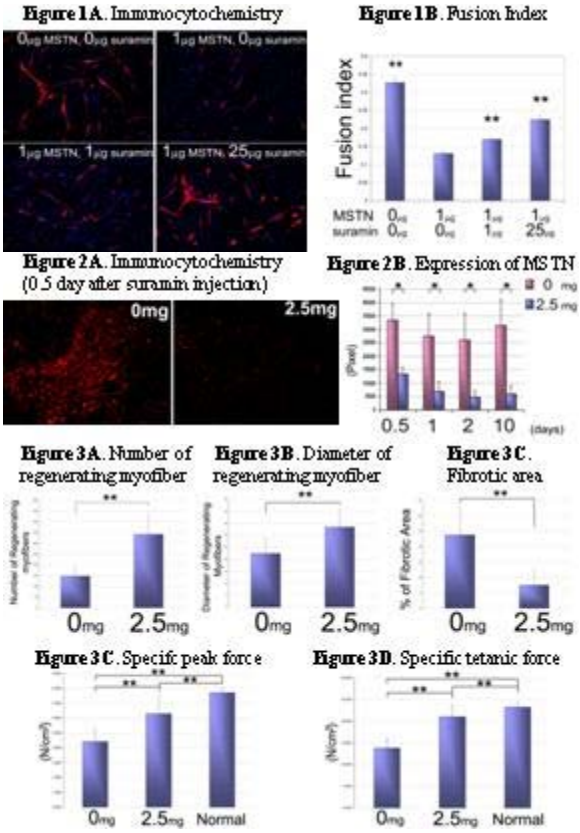
Animal model: The muscle contusion model was developed in tibialis anterior muscle of normal wild-type mice. Different concentrations of suramin (0 and 2.5 mg in 20μl of Phosphate-buffered solution [PBS]) were injected intramuscularly two weeks after injury (five mice in each group). Cryostat sections of muscles were obtained and histologically stained (hematoxlin and eosin stain (H&E) and Masson's Trichrome stain) to evaluate the regeneration by counting the number of centronucleated regenerating myofibers and measuring fibrosis four weeks after injury. Specific peak force and specific tetanic force were mesured as physiological tests to evaluate functional recovery after muscle injury using same protocol as above (five mice in each group). Furthermore immunohistochemistry of MSTN was performed at different time point (0.5, 1, 2, 10 days after suramin (0 and 2.5 mg) injection) to evaluate the expression of MSTN (five mice in each group at each time point). Northern Eclipse software (Empix Image, Inc.) was used to quantify the total fibrotic area and expression of MSTN. Statistical analysis was performed with student's t-test or ANOVA. Statistical significance was defined as $P < .05$ (*: P<.05, **: P<.01).

RESULTS:

Suramin blocked the proliferative effect of MSTN on fibroblasts and the inhibitory effect of MSDN of myoblast differentiation: MSTN treatment significantly promoted the proliferation on fibroblasts and the differentiation on myoblasts. However, suramin treatment significantly blocked both of those MSTN's effects and moreover suramin treatment stimulated the fusion on myoblasts in a dose-dependent manner in the presence of MSTN (Fig. 1A, B).

Suramin inhibits the myostatin expression in injured skeletal muscle: Suramin (2.5 mg) injection 2 weeks after contusion injury effectively inhibited the expression of MSTN when compared with the control group (0 mg) at different time points (0.5, 1, 2, 10 days after suramin injection) (Fig. 2A, B).

Suramin enhances muscle regeneration and decreases fibrosis and improves functional recovery after contusion injury: We observed a significant increase in the number and in the diameter of regenerating myofibers in the suramin treated group (2.5 mg) when compared with the control group (0mg) (Fig. 3A, B,). Moreover suramin treated group showed significantly less fibrotic area than control group (Fig. 3C). Furthermore the suramin treatment showed significant advantage in physiological evaluation (specific peak force and specific tetanic force) compared to control group.



DISCUSSION:

We have reported that suramin can effectively prevent muscle fibrosis and enhance muscle regeneration by blocking TGF-β1 after laceration and strain injury [2, 3]. Nevertheless, whether suramin would regulate the effect of MSTN, negative regulator of muscle growth, to improve muscle healing was still unknown. This is the first study to show that suramin down-regulates the proliferation on fibroblasts and up-regulates the differentiation on myoblasts by neutralizing MSTN. Moreover suramin injection in injured skeletal muscle effectively inhibited the expression of MSTN and enhanced the muscle regeneration and reduced the fibrosis *in vivo*. These results may reveal the hidden mechanism by which suramin improve the muscle healing after injuries. We have reported important anti-fibrotic molecules such as Decorin to improve injured skeletal muscles. However suramin has been already approved by FDA and this makes suramin more suitable agent for the therapy of muscle injury. Our findings may contribute to the development of biological therapies for muscle injury.

ACKNOWLEDGEMENTS: The authors are grateful for technical assistance from Maria Branca, Jessica Tebbets, and Aiping Lu and for scientific editing support from David Humiston . Funding support was provided by a grant from the Department of Defense (W81XWH-06-1-0406).

REFERENCES:

1. Li Y, Foster W *et al*., *Am J Pathol* 2004;164: 1007-19.
2. Chan Y, Li Y *et al*., *J Appl Physiol* 2003;95:771-80.
3. Chan Y, Li Y *et al*., *Am J Sports Med* 2005; 33:43-51.
4. Zhu J, , Li Y *et al*., *J Biol Chem* 2007, in print

The American Journal of Sports Medicine

http://ajs.sagepub.com/

Improved Muscle Healing After Contusion Injury by the Inhibitory Effect of Suramin on Myostatin, a Negative Regulator of Muscle Growth

Masahiro Nozaki, Yong Li, Jinhong Zhu, Fabrisia Ambrosio, Kenji Uehara, Freddie H. Fu and Johnny Huard

Am J Sports Med 2008 36: 2354 originally published online August 25, 2008
DOI: 10.1177/0363546508322886

The online version of this article can be found at:
http://ajs.sagepub.com/content/36/12/2354

Published by:
SAGE
http://www.sagepublications.com

On behalf of:
AOSSM
American Orthopaedic Society for Sports Medicine

Improved Muscle Healing After Contusion Injury by the Inhibitory Effect of Suramin on Myostatin, a Negative Regulator of Muscle Growth

Masahiro Nozaki,*† MD, Yong Li,*† MD, PhD, Jinhong Zhu,†‡ MD, Fabrisia Ambrosio,*†§ PhD MPT, Kenji Uehara,*† MD, PhD, Freddie H. Fu,* MD, and Johnny Huard,*†‡§ PhD
*From the *Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania, the †Stem Cell Research Center, Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania, and the ‡Department of Bioengineering and §Department of Physical Medicine and Rehabilitation, University of Pittsburgh, Pittsburgh, Pennsylvania*

Background: Muscle contusions are the most common muscle injuries in sports medicine. Although these injuries are capable of healing, incomplete functional recovery often occurs.

Hypothesis: Suramin enhances muscle healing by both stimulating muscle regeneration and preventing fibrosis in contused skeletal muscle.

Study Design: Controlled laboratory study.

Methods: In vitro: Myoblasts (C2C12 cells) and muscle-derived stem cells (MDSCs) were cultured with suramin, and the potential of suramin to induce their differentiation was evaluated. Furthermore, MDSCs were cocultured with suramin and myostatin (MSTN) to monitor the capability of suramin to neutralize the effect of MSTN. In vivo: Varying concentrations of suramin were injected in the tibialis anterior muscle of mice 2 weeks after muscle contusion injury. Muscle regeneration and scar tissue formation were evaluated by histologic analysis and functional recovery was measured by physiologic testing

Results: In vitro: Suramin stimulated the differentiation of myoblasts and MDSCs in a dose-dependent manner. Moreover, suramin neutralized the inhibitory effect of MSTN on MDSC differentiation. In vivo: Suramin treatment significantly promoted muscle regeneration, decreased fibrosis formation, reduced myostatin expression in injured muscle, and increased muscle strength after contusion injury.

Conclusion: Intramuscular injection of suramin after a contusion injury improved overall skeletal muscle healing. Suramin enhanced myoblast and MDSC differentiation and neutralized MSTN's negative effect on myogenic differentiation in vitro, which suggests a possible mechanism for the beneficial effects that this pharmacologic agent exhibits in vivo.

Clinical Relevance: These findings could contribute to the development of biological treatments to aid in muscle healing after experiencing a muscle injury.

Keywords: muscle contusion injury; suramin; myostatin; muscle regeneration; fibrosis

Muscle injuries are common musculoskeletal problems encountered in sports medicine clinics. Muscle contusion, produced by the impact of a nonpenetrating object,[8] is one of the most common muscle injuries.[3] Current therapies including RICE (rest, ice, compression, and elevation), immobilization, as well as active and passive range of motion exercise are the norm for treatment; however, complications such as muscle atrophy, contracture formation, and pain leading to functional and structural deficits often occur after severe muscle injury.[8,24,25,38] Optimal treatment strategies have not yet been clearly defined.

We have observed that the healing process of injured skeletal muscle in animal models consists of 3 distinct phases:

‖Address correspondence to Johnny Huard, PhD, 4100 Rangos Research Center, 3460 Fifth Avenue, Pittsburgh, PA, 15213-2582 (e-mail: jhuard@pitt.edu).

No potential conflict of interest declared.

The American Journal of Sports Medicine, Vol. 36, No. 12
DOI: 10.1177/0363546508322886

degeneration and inflammation, regeneration, and fibrosis.[13,15,16,26] The first phase, which occurs in the first few days after injury, is characterized by local swelling at the injury site, the formation of a hematoma, necrosis of muscle tissue,[20,23] degeneration, and an inflammatory response, which consists of the infiltration of activated macrophages and T-lymphocytes into the injured tissue. The next phase, regeneration, usually occurs 5 to 10 days after injury and includes phagocytosis of the damaged tissue and regeneration of the injured muscle. This phase is promoted by the release of several growth factors that regulate myoblast proliferation and differentiation, produce connective tissue and scar tissue, and induce capillary ingrowth at the injury site.[1,4,17]

The final phase, the formation of scar tissue (fibrosis), usually begins between the second and third week after injury. The formation of scar appears to be the end product of the muscle repair process and hinders full muscle regeneration. We have previously reported that transforming growth factor–beta 1 (TGF-β1) is a major factor in triggering the fibrotic cascade within injured skeletal muscle.[30,31] With this in mind, we have focused on the use of antifibrotic agents (such as decorin and γ-interferon) that inhibit TGF-β1 expression, reduce scar tissue formation, and consequently improve muscle healing after injury.[12,13] Administration of decorin after muscle injury showed improvement of muscle healing both histologically and physiologically.[13] The injection of γ-interferon into injured skeletal muscle also showed similar effects.[12] Despite these compelling findings, which were derived from various murine injury models, the fact that decorin is not clinically available limits its translation to human subjects and γ-interferon demonstrates serious side effects in spite of US Food and Drug Administration (FDA) approval. A recently published study has shown that myostatin (MSTN), a member of the TGF-β superfamily and a negative regulator of muscle growth, stimulates scar tissue formation after skeletal muscle injury in vivo.[48] It was also shown in this study that decorin could inhibit MSTN's activity in vitro.[48] Because both decorin and suramin have the ability to inhibit TGF-β1 activity,[5,6,13] we hypothesize that suramin may also possess a similar anti-MSTN activity as decorin.

Suramin, an antiparasitic and antineoplastic agent, can inhibit TGF-β1's ability to bind to its receptors and has been shown to enhance muscle regeneration after strain and laceration injuries.[5,6] We pursued the use of suramin due to the fact that it is currently FDA-approved and could thereby be used clinically more readily than decorin. In the current study, we examine whether suramin can promote differentiation of myoblasts and muscle-derived stem cells (MDSCs) and neutralize the effect of MSTN in vitro. We also investigate whether suramin can improve muscle healing after muscle contusion, a common muscle injury, by enhancing regeneration and reducing fibrosis in vivo.

MATERIALS AND METHODS

In Vitro Potential of Suramin to Induce Myogenic Differentiation

Effect of Suramin on C2C12 Myoblasts. C2C12 cells, a well-known myoblast cell line, were cultured with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, California) containing 10% fetal bovine serum (FBS) (Invitrogen), and 1% penicillin/streptomycin (P/S) (Invitrogen). Cells were plated at a density of 10 000 cells/well into 12-well plates. After a 24-hour incubation period, the medium was completely removed and low serum-containing medium (DMEM, 2% horse serum [HS] [Invitrogen], and 1% P/S) was added with varying concentrations of suramin (0, 0.25, 2.5, 25 μg/mL) (Sigma, St Louis, Missouri). Medium was replaced with fresh medium (containing the same concentrations of suramin) every 2 days. All the cells were grown at 37°C in 5% CO_2 for a total of 4 days.

Effect of Suramin on MDSCs Isolated From Skeletal Muscle. MDSCs were isolated from 3-week-old male mice (C57BL10J+/+) via the previously described modified preplate technique.[31,35,36] Muscle-derived stem cells were cultured in proliferation medium (PM) containing DMEM, 10% FBS, 10% HS, 1% P/S, and 0.5% chick embryo extract (Sera Laboratories International, West Sussex, United Kingdom). Cells were plated at a density of 10 000 cells/well into 12-well plates. After a 24-hour incubation period, the medium was completely removed and low-serum medium was added with different concentrations of suramin (0, 1, 10, 100 μg/mL) for the next 24 hours. Medium was replaced with fresh low-serum medium for 1 more day. All the cells were grown at 37°C in 5% CO_2 for a total of 3 days.

Ability of Suramin to Neutralize MSTN's Inhibition of MDSC Differentiation. Muscle-derived stem cells were cultured in PM and plated at a density of 5000 cells/well into 24-well plates. After a 24-hour incubation period, the medium was removed and low serum-containing medium was added with varying concentrations of suramin (0, 1, 10, or 100 μg/mL) and MSTN (0 or 100 ng/mL) for the next 24 hours. Medium was replaced with fresh low serum medium containing the same concentrations of MSTN for 1 additional day. All the cells were grown at 37°C in 5% CO_2 for a total of 3 days.

Immunocytochemistry

To quantify the differentiation of the C2C12 cells and MDSCs, cells were fixed in cold methanol for 2 minutes and washed in Dulbecco's phosphate-buffered saline (PBS) for 10 minutes at room temperature (RT). Samples were washed 3 times in PBS, then incubated in blocking buffer (10% HS) for 30 minutes at RT. Cells were incubated overnight at 4°C with primary antibodies (monoclonal antiskeletal myosin [fast] clone MY-32 [Sigma]) in 2% HS. After washing in PBS, samples were incubated with the secondary antibody (goat antimouse IgG conjugated with Cy3 [Sigma]) in 2% HS for 1 hour at RT. The cell nuclei were stained with a Hoechst 33258 dye for 10 minutes at RT. Fusion index (ratio of nuclei in myotubes to all nuclei) was calculated to evaluate the myogenic differentiation capacity of cells.

Evaluation of the Histologic and Physiologic Effects of Suramin on Muscle Healing After Contusion Injury In Vivo

Animal Model. The policies and procedures followed for the animal experimentation performed in these studies are

in accordance with those detailed by the US Department of Health and Human Services and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.* Our Institutional Animal Care and Use Committee approved the research protocol used for these experiments (protocol No. 19/05). An animal model of muscle contusion was developed in normal mice (C57BL6J+/+, Jackson Laboratory, Bar Harbor, Maine) based on previously described studies.[8,26] Thirty-three mice, aged 8 to 10 weeks with weights of 21.0 to 26.3 g, were used in this experiment.

The mice were anesthetized with 1.0% to 1.5% isoflurane (Abbott Laboratories, North Chicago, Illinois) in 100% O_2 gas. The mouse's hindlimb was positioned by extending the knee and plantar flexing the ankle 90°. A 16.2-g, 1.6-cm stainless steel ball (Small Parts Inc, Miami Lakes, Florida) was dropped from a height of 100 cm onto the impactor that hit the mouse's tibialis anterior (TA) muscle. The muscle contusion made by this method was a high-energy blunt injury that created a large hematoma and was followed by massive muscle regeneration,[8,26] healing processes that are very similar to those seen in humans.[10] The mice were divided into 4 groups (6 mice/group) on the basis of the different concentrations of suramin to be injected (0, 2.5, 5, and 10 mg in 20 μL of PBS) after creating the contusion injury. Suramin was injected 2 weeks after injury. All animals were sacrificed to evaluate the healing histologically and physiologically at 4 weeks after injury. Three mice (6 muscles) per group were assessed histologically, 3 mice (6 muscles) per group were assessed physiologically, and 3 mice were used as normal controls in the physiologic tests.

Furthermore, 2 concentrations of suramin (0 and 2.5 mg in 20 μL of PBS) were injected 2 weeks after injury (3 mice/group). The mice of both groups were sacrificed 2 days after injection for histologic analysis of MSTN expression.

Evaluation of Muscle Regeneration After Suramin Therapy. Tibialis anterior muscles that were used for histologic evaluation were isolated and frozen in 2-methylbutane precooled in liquid nitrogen and subsequently cryosectioned. Hematoxylin and eosin staining was done to monitor the number of regenerating myofibers within the injury sites treated with suramin (0, 2.5, 5, or 10 mg in 20 μL of PBS), and the results were compared among the different groups. Centronucleated myofibers were considered to be regenerating myofibers. Centrally nucleated myofibers are a sign of regeneration in injured and diseased muscle. Upon myoblast fusing into myotubes or with myofibers, the nuclei remain in the endomysial tube found in the center of the myofiber until it has reached maturity whereby the nuclei migrate to their final positions at the periphery of the mature fiber in the subsarcolemmal position. The central nucleation is easily identified by hematoxylin and eosin or trichrome staining.[11,37] Analysis of regenerating myofibers was performed using Northern Eclipse software (Empix Imaging Inc, Cheektawaga, New York). The total number of regenerating myofibers within the injury site was quantified by using 10 random fields selected from each sample in accordance with a previously described protocol.[12,13,26,33]

Evaluation of Fibrosis After Suramin Therapy. To measure areas of fibrotic tissue in the injury sites, Masson's trichrome staining (IMEB Inc, Chicago, Illinois) was performed. After Masson trichrome staining, the ratio of the fibrotic area to the total cross-sectional area was calculated to estimate the fibrosis formation by using Northern Eclipse software (Empix Imaging Inc). The ratio of the fibrotic area within the injury sites was quantified using a previously described protocol.[12,13,26, 3]

Evaluation of MSTN Expression After Suramin Therapy. To measure the MSTN expression in injured muscle, cryosectioned tissue was fixed in 4% formalin for 5 minutes followed by two 10-minute washes with PBS. The sections were then blocked with 10% HS for 1 hour at RT. Sections were incubated overnight at 4°C with primary antibodies (goat anti GDF-8 AF-788, R&D Systems Inc, Minneapolis, Minnesota) in 2% HS. After washing in PBS, samples were incubated with the secondary antibody (donkey anti-goat IgG conjugated with Alexa Fluor 555 [Invitrogen]) in 2% HS for 1 hour at RT.

The total MSTN positive area was calculated using Northern Eclipse software (Empix Imaging Inc) The total positive area within the injury site was quantified using a previously described protocol.[12,13,26,33]

Physiologic Evaluation of Muscle Contractile Properties After Suramin Therapy. Contusion injuries were made on TA muscles of both legs of normal mice and treated as described above. Four weeks after injury, muscles from both legs were tested to evaluate peak twitch and tetanic force. Tibialis anterior muscles were harvested bilaterally, placed in a vertical chamber that was constantly perfused with mammalian Ringer solution aerated with 95% O_2–5% CO_2 and maintained at 25°C. The distal attachment of the muscle was mounted to a glass tissue-support rod, and the proximal end of muscle on the tibia was connected to a force transducer and length servo system (Aurora Scientific, Aurora, Ontario, Canada). The muscles were stimulated by monophasic rectangular pulses of current (1 millisecond in duration) delivered through platinum electrodes placed 1 cm apart. The current was increased by 50% more than the current necessary to obtain peak force (250-300 mA) to ensure maximal stimulation. Using a micropositioner, muscles were first adjusted to their optimum length (Lo), defined as the length at which maximum isometric twitch tension was produced. Maximal tetanic force was assessed via a stimulation frequency of 75 Hz delivered in a 500-millisecond train. After the procedure, each muscle was weighed and specific peak twitch force and specific peak tetanic force were calculated and expressed in force per unit cross-sectional area (N/cm^2).

Statistical Analysis

All of the results from this study were expressed as the mean ± standard deviation. The result of MSTN expression in vivo was analyzed using the *t* test and all the other results were statistically analyzed using analysis of variance. Differences among the groups were analyzed by using Scheffé multiple comparisons (post hoc test). Statistical significance was defined as $P < .05$.

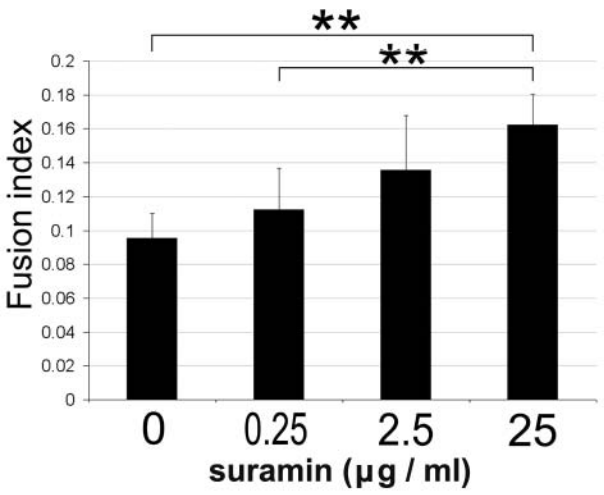


Figure 1. Fusion index was measured to evaluate C2C12 myoblast differentiation stimulated by different concentration of suramin (0, 0.25, 2.5, and 25 µg/mL). $^{**}P < .01$.

RESULTS

In Vitro Potential of Suramin to Induce Myogenic Differentiation

Effect of Suramin on Myoblasts. The effect of suramin on the differentiation of C2C12 cells is shown in Figure 1. The fusion index of C2C12 cells in the group treated with suramin (25 µg/mL) (0.162 ± 0.018) was significantly higher at 4 days of incubation as compared with the fusion index in the control group (0 µg/mL) (0.953 ± 0.015). Moreover, the high-dose suramin-treated group (25 µg/mL) displayed a significantly higher fusion index than the low-dose suramin-treated group (0.25 µg/mL). The effect of suramin to induce the differentiation of C2C12 appeared to be dose-dependent.

Effect of Suramin on MDSCs. Suramin also stimulated MDSC differentiation in a dose-dependent manner (Figure 2E). Suramin-treated MDSCs enhanced the differentiation of MDSCs after 3 days of incubation. Suramin-treated groups (10 and 100 µg/mL) (Figures 2C and 2D) showed significantly higher fusion indexes (0.228 ± 0.025, 0.347 ± 0.0456) than the control group (0 µg/mL) (Figure 2A) (0.149 ± 0.030). Among the suramin-treated groups, the high-dose suramin-treatment group (100 µg/mL) (Figure 2D) displayed a significantly higher fusion index when compared with the 2 lower-dose treatment groups (1 and 10 µg/mL) (Figures 2B and 2C).

Ability of Suramin to Neutralize MSTN's Inhibition of MDSC Differentiation. The group containing only MSTN (0 µg/mL suramin and 100 ng/mL MSTN) (Figure 3B) showed a significantly lower fusion index (0.091 ± 0.020) than the control group (0 µg/mL suramin and 0 ng/mL MSTN) (Figure 3A) (0.152 ± 0.035). All groups containing both suramin and MSTN (1, 10, and 100 µg/mL suramin and 100 ng/mL MSTN) (Figures 3C, 3D, and 3E) demonstrated significantly higher fusion indexes (0.153 ± 0.035, 0.162 ± 0.048, 0.279 ± 0.041) when compared with the group containing only MSTN (Figure 3B). Suramin appeared to neutralize MSTN's inhibitory effect on MDSC differentiation and, moreover, appeared to stimulate the fusion of MDSCs in a dose-dependent manner (Figure 3F).

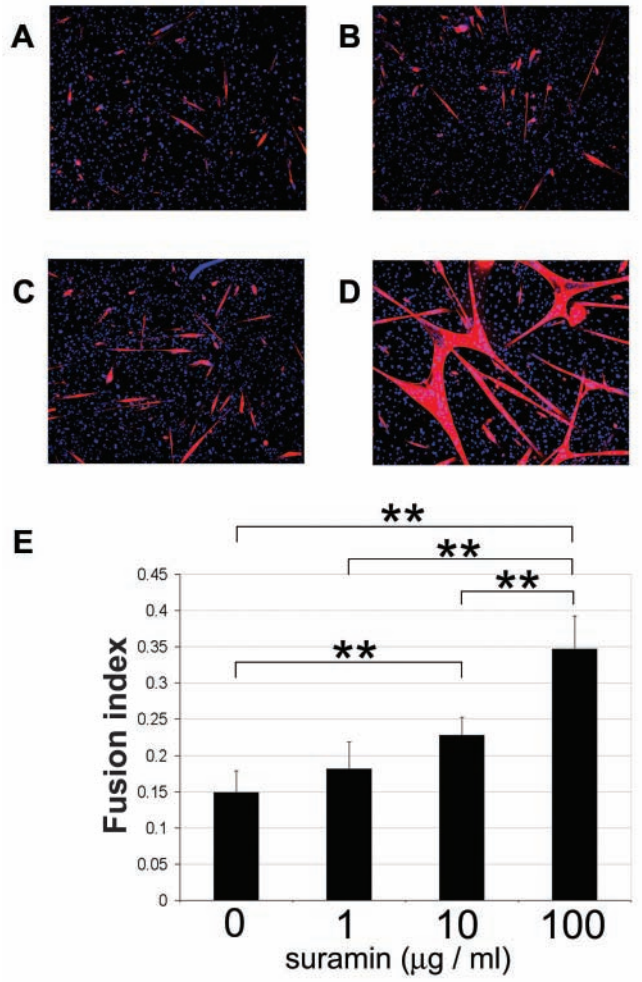


Figure 2. Immunocytochemical staining of muscle-derived stem cells (MDSCs) for fast myosin heavy chain at 3 days after incubation with different doses of suramin (A, 0 µg/mL; B, 1 µg/mL; C, 10 µg/mL; and D, 100 µg/mL). Myotubes are shown in red and nuclei are in blue (original magnification, ×200). E, comparison of fusion index of MDSC differentiation. $^{**}P < .01$.

Histologic and Physiologic Effects of Suramin on Muscle Healing After Contusion Injury In Vivo

Effect of Suramin Treatment on Muscle Regeneration After Contusion Injury. All the centronucleated regenerating myofibers present in the injured muscle were counted and compared among the groups (Figure 4E). The suramin-treated muscles displayed numerous regenerating myofibers at the contusion site. All the suramin-treated groups (2.5, 5, and 10 mg) (Figures 4B, 4C, and 4D) showed significantly higher numbers of regenerating myofibers (361.6 ± 84.39, 341.1 ± 57.21, 308.5 ± 84.33)

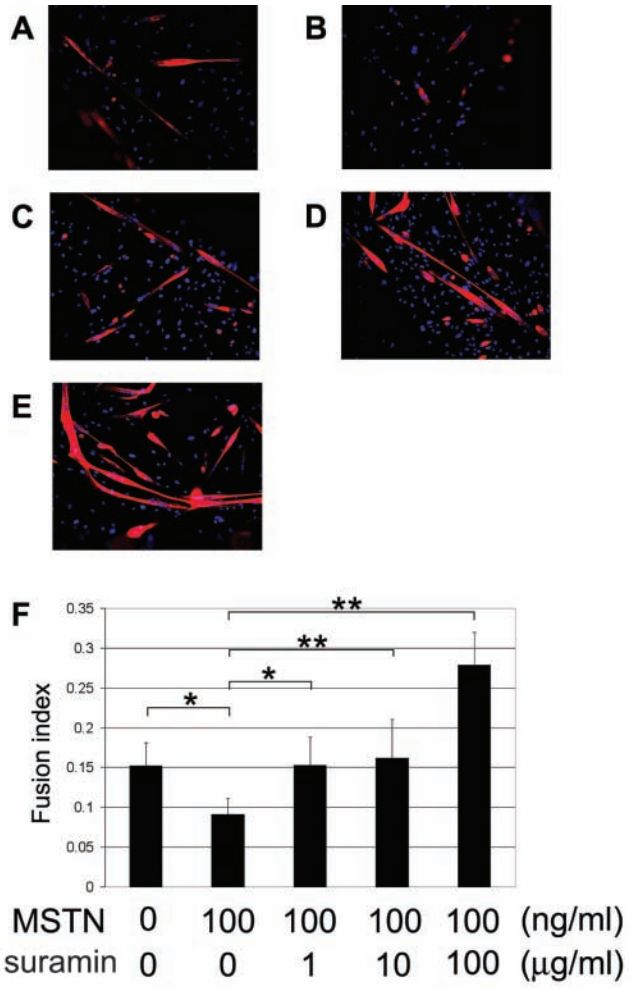


Figure 3. Immunocytochemical staining of muscle-derived stem cells (MDSCs) for fast myosin heavy chain at 3 days after incubation in 2% Dulbecco's modified Eagle's medium with different doses of suramin and myostatin (MSTN) (A, 0 μg/mL suramin and 0 ng/mL MSTN; B, 0 μg/mL suramin and 100 ng/mL MSTN; C, 1 μg/mL suramin and 100 ng/mL MSTN; D, 10 μg/mL suramin and 100 ng/mL MSTN; and E, 100 μg/mL suramin and 100 ng/mL MSTN). Myotubes are shown in red and nuclei are in blue (original magnification, ×200). F, comparison of fusion index of MDSC differentiation. *$P < .05$, **$P < .01$.

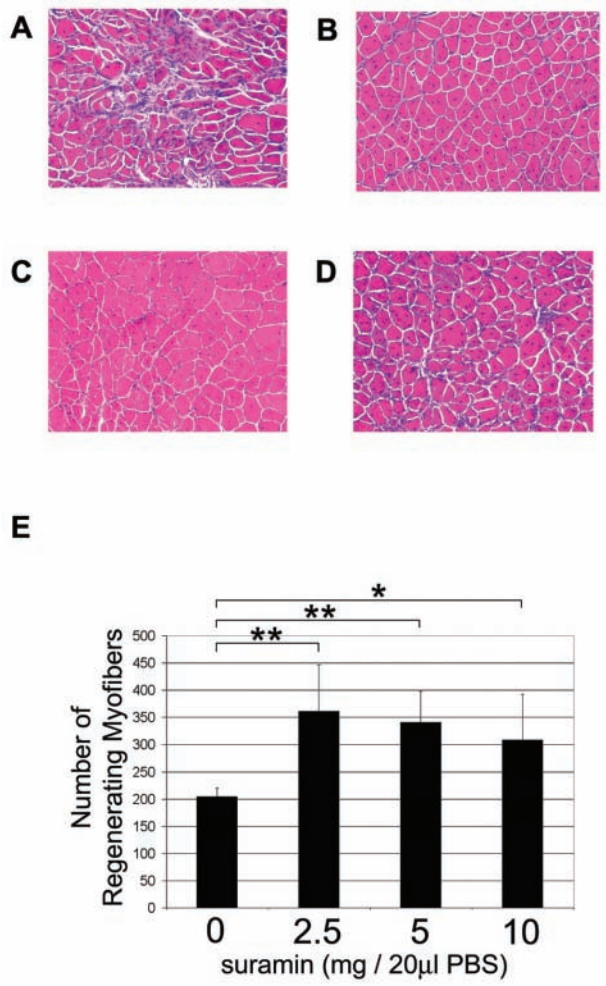


Figure 4. Histologic evaluation of muscle regeneration at four weeks after contusion injury by hematoxylin and eosin staining of tibialis anterior muscle treated with different concentrations of suramin (A, 0 mg/20 μL phosphate-buffered saline [PBS]; B, 2.5 mg/20 μL PBS; C, 5 mg/20 μL PBS; and D, 10 mg/20 μL PBS) injected at 2 weeks after injury. Regenerating myofibers were defined by centronucleated myofibers (original magnification, ×100). E, quantification of the number of regenerating myofibers treated with various concentrations of suramin. *$P < .05$, **$P < .01$.

compared with the control group (0 mg of suramin) (Figure 4A) (204.6 ± 15.27).

Effect of Suramin Therapy on Muscle Fibrosis After Contusion Injury. After Masson trichrome staining, the area of fibrotic scar tissue was evaluated and compared among the groups (Figure 5E). All the suramin-treated groups (2.5, 5, and 10 mg) (Figures 5B, 5C, and 5D) showed significantly less fibrotic area (6.856 ± 2.588, 7.677 ± 2.897, 8.993 ± 2.980) compared with the untreated control group (0 mg of suramin) (Figure 5A) (19.109 ± 3.215).

Suramin Injection Downregulated MSTN Expression in Injured Muscle. Immunohistochemical staining was performed to detect MSTN expression in the contusion-injured TA muscles. The MSTN-positive areas were measured and compared between the various treatment groups (Figure 6C). The muscle treated with 2.5-mg suramin injection 2 weeks after injury showed significantly less MSTN expression (4466.7 ± 7306.1) when compared with the untreated control (0 mg suramin) (27 830.2 ± 23 206.3) (Figures 6A and 6B).

Suramin Injection Improved Muscle Strength After Contusion Injury. The results of the physiologic evaluations are shown in Table 1. The control group (0 mg of suramin) and all the suramin-treatment groups, with the exception of the 2.5-mg suramin-treatment group, showed significantly less specific peak force (twitch and tetanic) when compared with the normal noninjured group. On the other hand, the muscles treated with 2.5 mg of suramin

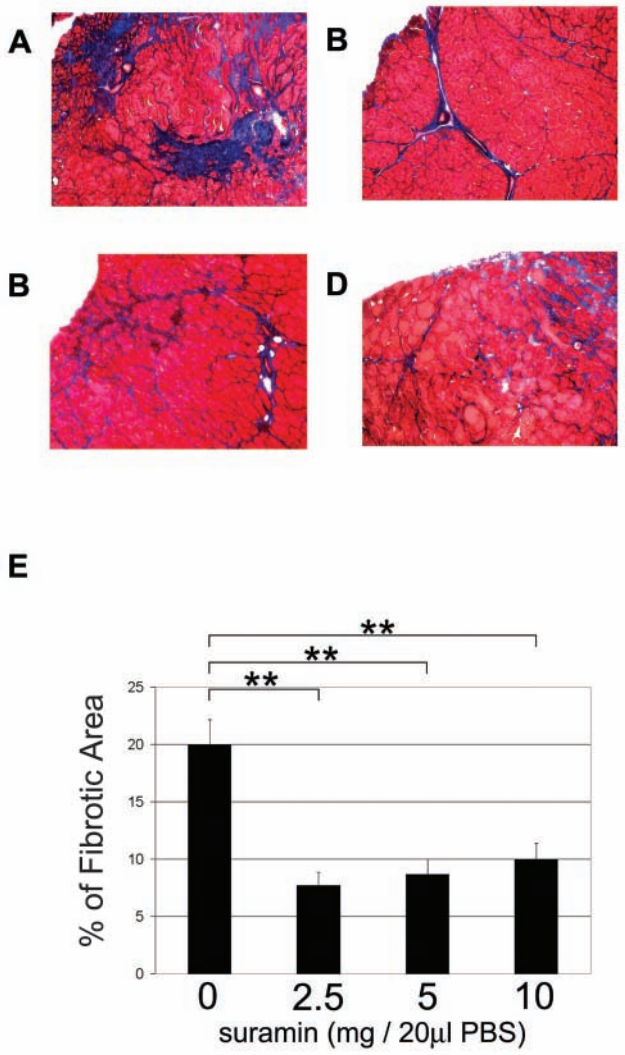


Figure 5. Histologic evaluation of the formation of scar tissue at 4 weeks after contusion injury by Masson trichrome staining of tibialis anterior muscle treated with different concentrations of suramin (A, 0 mg/20 µL phosphate-buffered saline [PBS]; B, 2.5 mg/20 µL PBS; C, 5 mg/20 µL PBS; and D, 10 mg/20 µL PBS) injected at 2 weeks after injury. Scar tissues are shown in blue and muscles are in red (original magnification, ×100). E, quantification of the scar tissue area in tibialis anterior muscle treated with various concentrations of suramin. $^{**}P < .01$.

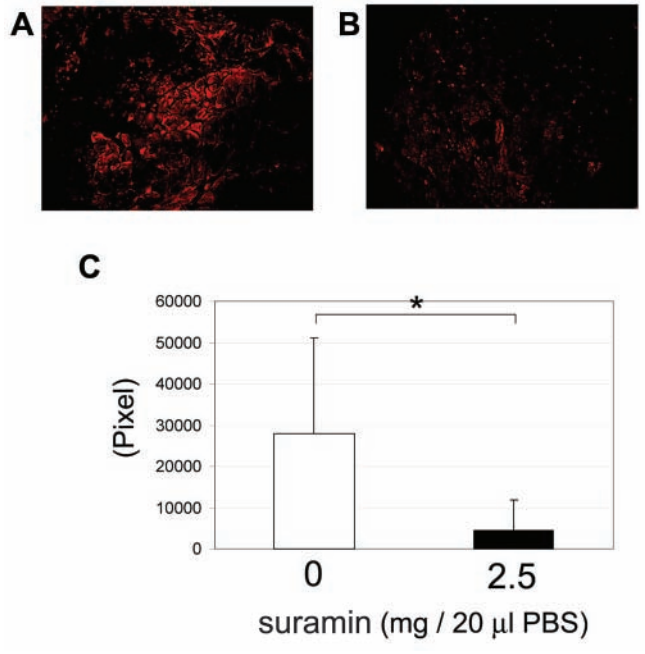


Figure 6. Immunohistochemical staining for MSTN at 2 days after suramin injection 2 weeks after injury (A, 0 mg suramin/20 µL phosphate-buffered saline [PBS]; B, 2.5 mg suramin/20 µL PBS). C, quantification of the total MSTN-positive area treated with suramin. $^{*}P < .05$.

TABLE 1
Results of Specific Peak Twitch and Titanic Force

Group	Specific Peak Twitch Force (N/cm^2)	Specific Peak Titanic Force (N/cm^2)
Normal (noninjured)	7.728 ± 0.312^a	23.272 ± 1.334^a
0 mg suramin	4.431 ± 0.903	13.797 ± 2.136
2.5 mg suramin	6.305 ± 1.239^b	21.055 ± 2.662^a
5 mg suramin	5.014 ± 0.337	15.131 ± 0.830
10 mg suramin	4.993 ± 1.074	15.085 ± 2.792

$^a P < .01$ compared with 0, 5, and 10 mg suramin groups.
$^b P < .05$ compared with 0 mg suramin group.

showed significantly greater specific peak force (twitch and tetanic) than the untreated control group. There was no significant difference between injured muscle that had been treated with 2.5 mg of suramin and normal noninjured muscle. In addition, the muscles injected with 2.5 mg of suramin also showed significantly greater specific peak tetanic force than the other suramin-treated groups (5 and 10 mg) and the control group (0 mg of suramin).

DISCUSSION

The aim of this study was to evaluate the effect of suramin on myogenic cell differentiation and muscle healing after contusion injury. We hypothesized that suramin treatment would lead to better biological healing by both stimulating muscle regeneration and preventing fibrosis in contused muscle. Histologically, the muscle treated with suramin 2 weeks after injury showed more regenerating myofibers and less fibrotic scar formation when compared with the control group (0 mg of suramin) at 4 weeks after contusion injury. Furthermore, suramin treatment also showed an increase in muscle strength compared with the untreated control group.

The muscle contusion injuries we modeled in this study are among the most common muscle injuries encountered in contact sports and by military personnel. More than

90% of muscle injuries are caused either by contusion or by excessive strain of the muscle.[3,14] Although contusion injury is capable of healing, incomplete functional recovery often occurs, depending on the severity of the initial trauma. Skeletal muscle has a great regenerative potential,[†] largely attributed to the activation of muscle progenitor cells and their fusion into mature multinucleated myofibers[22,40,41]; however, scar tissue formation occurs simultaneously and likely competes with muscle regeneration during the muscle-healing process.[12,26,32]

We have investigated the effect of some treatments, such as suture or immobilization, which are normally used in the clinic for muscle injury, by using animal models. One study showed that immobilization after muscle laceration had no significant effect on fibrosis reduction. Suture repair promoted better healing of the injured muscle and prevented the development of fibrosis at the deep-tissue level, but not superficially.[33]

Suramin, which is a polysulphonated naphthylurea, was designed as an antiparasitic drug[18] and is used for the treatment of human sleeping sickness, onchocerciasis, and other diseases caused by trypanosomes and other worms. In addition, it is under investigation as a treatment for some malignancies such as prostate, adrenal cortex, lymphoma, breast, and colon cancers[49] and for human immunodeficiency virus–1.[29] The major systemic side effects of suramin are malaise, neuropathy,[2,7] mineral corticoid insufficiency,[27] and corneal deposits[19]; occasionally neutropenia,[9] thrombocytopenia,[47] and renal failure[42] have also been observed. The toxicity of suramin when injected intramuscularly, as we did in our animal model, has not yet been determined. Although we observed no adverse effects in the mice injected with up to 10 mg of suramin in this study, we did not specifically test to evaluate side effects. Suramin is known as a heparin analog that can bind to heparin-binding proteins and inhibit the effect of growth factors by competitively binding to growth factor receptors.[43,49] Transforming growth factor-β1, -β2, and -β3; platelet-derived growth factor (PDGF) A and B; and epidermal growth factor are growth factors that are known to be inhibited by suramin. Among them, TGF-β1 and -β2 and PDGF A and B are known to have the potential to promote fibroblast proliferation.[44,46]

In our in vivo study using the muscle-contusion model, direct injection of suramin at 2 weeks after muscle injury demonstrated a significant reduction of fibrous tissue formation when compared with the untreated control (0 mg of suramin). We found that suramin treatment led to a beneficial effect in contused muscle, as was previously seen in suramin-treated lacerations and strains[5,6] as well as when using other antifibrotic agents such as decorin and interferon gamma.[12,13] Moreover, our in vivo results indicate that 2.5 mg of suramin was the optimal dosage for the treatment of muscle contusions as there was no significant difference observed when higher dosages (5 and 10 mg) of suramin was injected into the injury site. Our previous work with the laceration and strain models compared doses that were different than the current study and we have difficulty making direct comparisons between the current study and the former studies. In the laceration study, we compared suramin doses at 0.25, 1.0, and 2.5 mg and found that the 2.5-mg dose was optimal.[6] In the strain model study, we compared doses at 0.25, 1.0, and 5 mg and found that the 5-mg dose was optimal.[5] Because we did not use a 2.5-mg dose in the strain study and did not use a 5-mg dose in the laceration study, we cannot definitively say that the 2.5-mg dosage would be optimal for these types of injuries, although it is strongly inferred.

This study also showed that suramin enhanced muscle regeneration when it was injected directly into the muscle 2 weeks after receiving a contusion injury. We found many regenerating myofibers at the injury site, usually sequestered by a large amount of fibrotic tissue. All the suramin-treated groups showed a significant increase in the number of regenerating myofibers at 4 weeks after receiving the injury.

Functional recovery after muscle injury is the most important variable determining the likelihood for clinical translation of this therapy in the treatment of skeletal muscle injury. As was observed with other antifibrotic agents (decorin and interferon gamma),[12,13] suramin also appears to have a beneficial effect on the physiologic recovery of skeletal muscle. Our results showed that there was no significant difference in the specific peak twitch and tetanic forces between normal noninjured muscle and contused 2.5-mg suramin-treated muscle. These results strongly indicate that the injection of 2.5 mg of suramin at 2 weeks after contusion injury can improve muscle strength and promote functional recovery after muscle injury.

Taken together, our in vitro study about the effect of suramin on myogenic cell differentiation provides insight as to the underlying mechanism by which suramin enhances muscle regeneration in vivo. Our in vitro results showed 25 μg/mL of suramin can significantly enhance C2C12 differentiation at 4 days after incubation and, in addition, suramin treatment leads to significant increases in the fusion index in a dose-dependent manner. Further, we observed an even more prominent dose-dependent effect on MDSC differentiation 3 days after initiating the incubation with suramin. Moreover, suramin also showed a neutralizing effect on MSTN, which inhibits differentiation of MDSCs in a dose-dependent manner.

We found that suramin stimulates myogenic differentiation in vitro. It is consistent with our in vivo results, showing that suramin is capable of enhancing muscle regeneration and improving muscle healing after muscle injury. Furthermore, as expected, we observed that MSTN significantly inhibits the myogenic differentiation of MDSCs; however, in the presence of suramin, the inhibitory effect of MSTN on myogenic differentiation was attenuated in a dose-dependent manner. These results suggest that suramin can neutralize MSTN activity. Therefore, we hypothesize that when myoblasts and MDSCs are treated with suramin, suramin stimulates myogenic differentiation by neutralizing the effect of endogenous MSTN. A similar neutralizing effect on MSTN has been seen when cells were treated with decorin, a TGF-β1 blocker.[48]

[†]References 4, 14, 16, 21, 26, 28, 33, 45

On the basis of these results, we further investigated whether suramin exerts a beneficial effect in the injured muscle through regulating MSTN expression. Not surprisingly, it was revealed that treatment with suramin significantly decreased MSTN expression in the injured muscle. Our previous study showed that MSTN can act with TGF-β1 to magnify fibrosis cascades in injured muscles.[48] Taken together, it may suggest that suramin administration effectively leads to enhanced muscle regeneration and reduced fibrosis after muscle injury via downregulation of endogenous MSTN.

This is the first study conducted to evaluate the effects that suramin would have on a contusion injury. It is also the first study to demonstrate that one of the mechanisms by which suramin could enhance skeletal muscle healing was by the downregulation of MSTN. Our results showed suramin can enhance myogenic cell differentiation and neutralize the effects of MSTN, which downregulates MDSC differentiation. Moreover, these in vitro results may reveal a possible mechanism by which suramin directly enhances muscle regeneration after muscle injury. Future studies should investigate further the mechanism by which suramin appears to stimulate myogenic differentiation and promotes muscle healing via MSTN regulation.

In summary, we demonstrated that the direct injection of suramin at 2 weeks after contusion injury can effectively reduce fibrotic scar formation and enhance muscle regeneration 4 weeks after injury. Physiologic evaluation also showed that suramin can enhance muscle functional recovery after contusion injury. Our in vitro study demonstrated that culturing either C2C12 myoblasts or MDSCs with suramin led to a significant increase in the fusion index of the cells. Moreover, with the addition of both suramin and MSTN in cell cultures, suramin was able to counteract MSTN's biological activity, thereby rescuing MSTN-inhibited myogenic differentiation of MDSCs.

The greatest advantage of using suramin is that this drug has already been approved by the FDA.[34,39] Future studies should consider the use of this agent for off-label use in the treatment of skeletal muscle injuries. Our findings could contribute to the development of progressive therapies for treating skeletal muscle injuries.

ACKNOWLEDGMENT

We thank Maria Branca, Jessica Tebbets, Aiping Lu, and Terry O'Day for technical assistance and James Cummins for editing the manuscript. Funding support was provided by the Department of Defense (W81XWH-06-1-0406, awarded to J.H.), the William F. and Jean W. Donaldson Chair at Children's Hospital of Pittsburgh, and the Henry J. Mankin Endowed Chair in Orthopaedic Surgery at the University of Pittsburgh.

REFERENCES

1. Alameddine HS, Dehaupas M, Fardeau M. Regeneration of skeletal muscle fibers from autologous satellite cells multiplied in vitro: an experimental model for testing cultured cell myogenicity. *Muscle Nerve.* 1989;12:544-555.
2. Bitton RJ, Figg WD, Venzon DJ, et al. Pharmacologic variables associated with the development of neurologic toxicity in patients treated with suramin. *J Clin Oncol.* 1995;13:2223-2229.
3. Canale ST, Cantler ED Jr, Sisk TD, Freeman BL. A chronicle of injuries of an American intercollegiate football team. *Am J Sports Med.* 1981;9:384-389.
4. Carlson BM, Faulkner JA. The regeneration of skeletal muscle fibers following injury: a review. *Med Sci Sports Exerc.* 1983;15: 187-198.
5. Chan YS, Li Y, Foster W, Fu FH, Huard J. The use of suramin, an antifibrotic agent, to improve muscle recovery after strain injury. *Am J Sports Med.* 2005;33:43-51.
6. Chan YS, Li Y, Foster W, et al. Antifibrotic effects of suramin in injured skeletal muscle after laceration. *J Appl Physiol.* 2003;95:771-780.
7. Chaudry V, Eisenberger MA, Sinibaldi VJ, Sheikh H, Griffin JW, Cornblath DR. A prospective study of suramin induced peripheral neuropathy. *Brain.* 1996;119:2039-2052.
8. Crisco JJ, Jokl P, Heinen GT, Connell MD, Panjabi MM. A muscle contusion injury model: biomechanics, physiology, and histology. *Am J Sports Med.* 1994;22:702-710.
9. Dawson NA, Lush RM, Steinberg SM, Tompkins AC, Headlee DJ, Figg WD. Suramin-induced neutropenia. *Eur J Cancer.* 1996;32A: 1534-1539.
10. Diaz JA, Fischer DA, Rettig AC, Davis TJ, Shelbourne KD. Severe quadriceps muscle contusions in athletes: a report of three cases. *Am J Sports Med.* 2003;31:289-293.
11. Fischman DA. The synthesis and assembly of myofibrils in embryonic muscle. *Curr Top Dev Biol.* 1970;5:235-280.
12. Foster W, Li Y, Usas A, Somogyi G, Huard J. Gamma interferon as an antifibrosis agent in skeletal muscle. *J Orthop Res.* 2003;21: 798-804.
13. Fukushima K, Badlani N, Usas A, Riano F, Fu F, Huard J. The use of an antifibrosis agent to improve muscle recovery after laceration. *Am J Sports Med.* 2001;29:394-402.
14. Garrett WE Jr. Muscle strain injuries: clinical and basic aspects. *Med Sci Sports Exerc.* 1990;22:436-443.
15. Garrett WE Jr, Safran MR, Seaber AV, Glisson RR, Ribbeck BM. Biomechanical comparison of stimulated and nonstimulated skeletal muscle pulled to failure. *Am J Sports Med.* 1987;15:448-454.
16. Garrett WE Jr, Seaber AV, Boswick J, Urbaniak JR, Goldner JL. Recovery of skeletal muscle after laceration and repair. *J Hand Surg [Am].* 1984;9:683-692.
17. Grounds MD. Towards understanding skeletal muscle regeneration. *Pathol Res Pract.* 1991;187:1-22.
18. Hawking F. Suramin: with special reference to onchocerciasis. *Adv Pharmacol Chemother.* 1978;15:289-322.
19. Hemady RK, Sinibaldi VJ, Eisenberger MA. Ocular symptoms and signs associated with suramin sodium treatment for metastatic cancer of the prostate. *Am J Ophthalmol.* 1996;121:291-296.
20. Honda H, Kimura H, Rostami A. Demonstration and phenotypic characterization of resident macrophages in rat skeletal muscle. *Immunology.* 1990;70:272-277.
21. Hughes C 4th, Hasselman CT, Best TM, Martinez S, Garrett WE Jr. Incomplete, intrasubstance strain injuries of the rectus femoris muscle. *Am J Sports Med.* 1995;23:500-506.
22. Hurme T, Kalimo H. Activation of myogenic precursor cells after muscle injury. *Med Sci Sports Exerc.* 1992;24:197-205.
23. Hurme T, Kalimo H, Lehto M, Jarvinen M. Healing of skeletal muscle injury: an ultrastructural and immunohistochemical study. *Med Sci Sports Exerc.* 1991;23:801-810.
24. Jackson DW, Feagin JA. Quadriceps contusions in young athletes: relation of severity of injury to treatment and prognosis. *J Bone Joint Surg Am.* 1973;55:95-105.
25. Jarvinen MJ, Lehto MU. The effects of early mobilisation and immobilisation on the healing process following muscle injuries. *Sports Med.* 1993;15:78-89.
26. Kasemkijwattana C, Menetrey J, Somogyl G, et al. Development of approaches to improve the healing following muscle contusion. *Cell Transplant.* 1998;7:585-598.

27. Kobayashi K, Weiss RE, Vogelzang NJ, Vokes EE, Janisch L, Ratain MJ. Mineralocorticoid insufficiency due to suramin therapy. *Cancer.* 1996;78:2411-2420.
28. Lefaucheur JP, Sebille A. Muscle regeneration following injury can be modified in vivo by immune neutralization of basic fibroblast growth factor, transforming growth factor beta 1 or insulin-like growth factor I. *J Neuroimmunol.* 1995;57:85-91.
29. Levine AM, Gill PS, Cohen J, et al. Suramin antiviral therapy in the acquired immunodeficiency syndrome: clinical, immunological, and virologic results. *Ann Intern Med.* 1986;105:32-37.
30. Li Y, Foster W, Deasy BM, et al. Transforming growth factor-beta1 induces the differentiation of myogenic cells into fibrotic cells in injured skeletal muscle: a key event in muscle fibrogenesis. *Am J Pathol.* 2004;164:1007-1019.
31. Li Y, Huard J. Differentiation of muscle-derived cells into myofibroblasts in injured skeletal muscle. *Am J Pathol.* 2002;161:895-907.
32. Menetrey J, Kasemkijwattana C, Day CS, et al. Growth factors improve muscle healing in vivo. *J Bone Joint Surg Br.* 2000;82:131-137.
33. Menetrey J, Kasemkijwattana C, Fu FH, Moreland MS, Huard J. Suturing versus immobilization of a muscle laceration: a morphological and functional study in a mouse model. *Am J Sports Med.* 1999;27:222-229.
34. Mietz H, Chevez-Barrios P, Feldman RM, Lieberman MW. Suramin inhibits wound healing following filtering procedures for glaucoma. *Br J Ophthalmol.* 1998;82:816-820.
35. Qu Z, Balkir L, van Deutekom JC, Robbins PD, Pruchnic R, Huard J. Development of approaches to improve cell survival in myoblast transfer therapy. *J Cell Biol.* 1998;142:1257-1267.
36. Rando TA, Blau HM. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. *J Cell Biol.* 1994;125:1275-1287.
37. Reznik M, Engel WK. Ultrastructural and histochemical correlations of experimental muscle regeneration. *J Neurol Sci.* 1970;11:167-185.
38. Ryan JB, Wheeler JH, Hopkinson WJ, Arciero RA, Kolakowski KR. Quadriceps contusions: West Point update. *Am J Sports Med.* 1991;19:299-304.
39. Schrell UM, Gauer S, Kiesewetter F, et al. Inhibition of proliferation of human cerebral meningioma cells by suramin: effects on cell growth, cell cycle phases, extracellular growth factors, and PDGF-BB autocrine growth loop. *J Neurosurg.* 1995;82:600-607.
40. Schultz E. Satellite cell behavior during skeletal muscle growth and regeneration. *Med Sci Sports Exerc.* 1989;21(5 Suppl):S181-186.
41. Schultz E, Jaryszak DL, Valliere CR. Response of satellite cells to focal skeletal muscle injury. *Muscle Nerve.* 1985;8:217-222.
42. Smith A, Harbour D, Liebmann J. Acute renal failure in a patient receiving treatment with suramin. *Am J Clin Oncol.* 1997;20:433-434.
43. Stein CA. Suramin: a novel antineoplastic agent with multiple potential mechanisms of action. *Cancer Res.* 1993;53(10 Suppl): 2239-2248.
44. Sullivan KM, Lorenz HP, Meuli M, Lin RY, Adzick NS. A model of scarless human fetal wound repair is deficient in transforming growth factor beta. *J Pediatr Surg.* 1995;30:198-202.
45. Taylor DC, Dalton JD Jr, Seaber AV, Garrett WE Jr. Experimental muscle strain injury: early functional and structural deficits and the increased risk for reinjury. *Am J Sports Med.* 1993;21:190-194.
46. Thomas DW, O'Neill ID, Harding KG, Shepherd JP. Cutaneous wound healing: a current perspective. *J Oral Maxillofac Surg.* 1995;53: 442-447.
47. Tisdale JF, Figg WD, Reed E, McCall NA, Alkins BR, Horne MK 3rd. Severe thrombocytopenia in patients treated with suramin: evidence for an immune mechanism in one. *Am J Hematol.* 1996;51:152-157.
48. Zhu J, Li Y, Shen W, et al. Relationships between transforming growth factor-beta1, myostatin, and decorin: implications for skeletal muscle fibrosis. *J Biol Chem.* 2007;282:25852-25863.
49. Zumkeller W, Schofield PN. Growth factors, cytokines and soluble forms of receptor molecules in cancer patients. *Anticancer Res.* 1995;15:343-348.

The American Journal of Sports Medicine

http://ajs.sagepub.com/

Angiotensin II Receptor Blockade Administered After Injury Improves Muscle Regeneration and Decreases Fibrosis in Normal Skeletal Muscle

Hany S. Bedair, Tharun Karthikeyan, Andres Quintero, Yong Li and Johnny Huard

Am J Sports Med 2008 36: 1548 originally published online June 11, 2008
DOI: 10.1177/0363546508315470

The online version of this article can be found at:
http://ajs.sagepub.com/content/36/8/1548

Published by:
SAGE
http://www.sagepublications.com

On behalf of:
AOSSM
American Orthopaedic Society for Sports Medicine

Angiotensin II Receptor Blockade Administered After Injury Improves Muscle Regeneration and Decreases Fibrosis in Normal Skeletal Muscle

Hany S. Bedair,* MD, Tharun Karthikeyan,* MD, Andres Quintero,* MD, Yong Li,*† MD, PhD, and Johnny Huard,*†‡ PhD

*From the *Department of Orthopaedic Surgery, University of Pittsburgh Medical Center, and the †Stem Cell Research Center, Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania*

Background: Several therapeutic agents have been shown to inhibit fibrosis and improve regeneration after injury in skeletal muscle by antagonizing transforming growth factor-β1. Angiotensin receptor blockers have been shown to have a similar effect on transforming growth factor-β1 in a variety of tissues.

Hypothesis: Systemic treatment of animals after injury of skeletal muscle with an angiotensin receptor blocker may decrease fibrosis and improve regeneration, mainly through transforming growth factor-β1 blockade, and can be used to improve skeletal muscle healing after injury.

Study Design: Controlled laboratory study.

Methods: Forty mice underwent bilateral partial gastrocnemius lacerations. Mice were assigned randomly to a control group (tap water), a low-dose angiotensin receptor blocker group (losartan, 0.05 mg/mL), or a high-dose angiotensin receptor blocker group (0.5 mg/mL). The medication was dissolved in tap water and administered enterally. Mice were sacrificed 3 or 5 weeks after injury, and the lacerated muscles were examined histologically for muscle regeneration and fibrosis.

Results: Compared with control mice at 3 and 5 weeks, angiotensin receptor blocker–treated mice exhibited a histologic dose-dependent improvement in muscle regeneration and a measurable reduction in fibrous tissue formation within the area of injury.

Conclusion: By modulating the response to local and systemic angiotensin II, angiotensin receptor blocker therapy significantly reduced fibrosis and led to an increase in the number of regenerating myofibers in acutely injured skeletal muscle. The clinical implications for this application of angiotensin receptor blockers are potentially far-reaching and include not only sports- and military-related injuries, but also diseases such as the muscular dystrophies and trauma- and surgery-related injury.

Clinical Relevance: Angiotensin receptor blockers may provide a safe, clinically available treatment for improving healing after skeletal muscle injury.

Keywords: fibrosis; angiotensin receptor blocker; TGF-β; muscle injury; muscle regeneration

Skeletal muscle injuries are among the most common complaints of patients seen by general medical physicians and also account for a large majority of patients seen in orthopaedic clinics.[2] Investigations have demonstrated that the natural history of muscle injury proceeds through a highly coordinated sequence of steps. Unfortunately, the regenerative capacity of injured skeletal muscle is limited; fibrotic tissue is a common end result that predisposes the muscle to recurrent injury and limits recovery of function.[16] Clinical experience reveals a high recurrence rate of skeletal muscle strain injuries among athletes, approaching 30% in some professional-level athletes.[32] Advancements in the identification of molecular events and cellular transformations after muscle injury have flourished; however, the clinical treatment of this common condition still relies on conventional therapies of rest, ice, and anti-inflammatory medications; these are of limited efficacy in preventing or

‡Address correspondence to Johnny Huard, 4100 Rangos Research Center, 3460 Fifth Avenue, Pittsburgh, PA 15213 (e-mail: jhuard@pitt.edu).

No potential conflict of interest declared.

The American Journal of Sports Medicine, Vol. 36, No. 8
DOI: 10.1177/0363546508315470

treating posttraumatic muscle fibrosis and in reducing the rate of reinjury.[1,18,33]

In our laboratory, we have investigated several biological agents that have proved to be of some benefit in altering the natural course of muscle injury. Specifically, we have focused our recent efforts on agents that inhibit muscle fibrosis via inhibition of transforming growth factor-β1 (TGF-β1), a key cytokine in the fibrotic signaling pathway in skeletal muscle.[21] Using decorin, suramin, relaxin, and gamma interferon, we have demonstrated that these therapies can decrease fibrosis and increase regeneration after skeletal muscle injury.[9,13,14,22,24] However, their use clinically is hampered by relatively severe side-effect profiles, lack of oral dosing formulations, and in some cases, lack of Food and Drug Administration (FDA) approval for use in humans.

Fibrosis is a pathologic process that is not unique to the skeletal muscle system. Observations have linked pathologic fibrosis in various organ systems to the local effects of a naturally occurring molecule, angiotensin II, an end product of the blood pressure–regulating renin-angiotensin system. The modulation of angiotensin II with angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers has demonstrated decreased fibrosis and improved function in liver, kidney, and lung tissue.[23,27,28,30] Injured cardiac muscle in disease entities such as congestive heart failure also demonstrates dysfunction related to fibrosis. Myocardium exposed to decreased levels of angiotensin II, either through the use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers (ARB), has also demonstrated measurably improved function.[15,31] Investigators have also observed a relationship between the modulation of angiotensin II and skeletal muscle. Patients treated with angiotensin-modulating medications for hypertension also displayed the unexpected side effect of decreased rates of muscle wasting and a reduction in the relative amount of adipose tissue within their musculature.[25] Moreover, elite athletes, particularly those in endurance sports, have also demonstrated findings consistent with inherent differences in their body's metabolism of angiotensin II, with decreased exposure resulting in improved skeletal muscle function.[25] We have observed an improvement in skeletal muscle regeneration postinjury after treatment with angiotensin blockade; this is a finding that, to our knowledge, had not been reported previously.[4] Elegant experiments have subsequently been reported that elucidate the mechanism by which angiotensin II receptor blockade modulates TGF-β1, which has also been implicated in the prevention of muscle regeneration in murine models of chronic myopathic disease.[10] The hypothesis of this study is that systemic delivery of an angiotensin II receptor blocker can enhance muscle regeneration and decrease fibrosis in a laceration model of normal skeletal muscle injury. This therapy may be an effective treatment when instituted postinjury, which is a clinically relevant scenario for physicians treating muscle injuries.

MATERIALS AND METHODS

In Vitro

The role of angiotensin II regarding myoblast and fibroblast proliferation was investigated using a series of standard proliferation experiments. National Institutes of Health (NIH) 3T3 cells (fibroblast cell line) and C2C12 myoblasts, purchased from American Type Culture Collection (ATCC) (Rockville, Maryland), along with primary muscle fibroblast isolates (PP2 cells) obtained through the previously published preplating technique, were chosen for analysis.[29] These cells were cultured with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, California) containing 10% fetal bovine serum, 10% horse serum, 0.5% chicken embryo extract, and 1% penicillin/streptomycin. All cells were cultured at 37°C in 5% CO_2. National Institutes of Health 3T3, C2C12, and PP2 cells were seeded onto different 6-well plates. Cells were cultured with medium (described above) and 0, 10^{-8}, 10^{-6}, or 10^{-4} mol/L of human angiotensin II (Sigma Chemical, St Louis, Missouri). Cell counts were performed at 24, 48, and 96 hours using a standard cell-counting chamber.

The supernatant solution from the PP2 cell culture was extracted after 24 and 48 hours of exposure to the various concentrations of angiotensin II. An enzyme-linked immunosorbent assay (ELISA) was performed in accordance with the manufacturer's recommendations (Sigma Chemical) to determine the level of TGF-β1 in the solution. National Institutes of Health 3T3 cells were harvested 48 hours after incubation with or without angiotensin II, and a western blot was performed. After lysation, the samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes used for immunostaining. Mouse anti-TGF-β1 type I antibodies (Sigma Chemical) were then applied. The horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, Illnois) were developed using SuperSignal West Pico Chemiluminescent substrate (Pierce), and positive bands were visualized on radiographic film. All results were analyzed using Northern Eclipse software v.6.0 (Empix Imaging, Mississauga, Ontario, Canada).

Animal Model

Forty immunocompetent mice (C57Bl/6J; Jackson Laboratory, Bar Harbor, Maine) were used for the histologic analysis. The animals were housed in cages and fed with commercial pellets. The policies and procedures of the animal laboratory were in accordance with those detailed by the US Department of Health and Human Services and the Animal Research Care Committee of the authors' institution (Protocol #25-03). A previously reported muscle laceration injury model was employed for these experiments, the healing process of which is similar histologically to strain injury models, which account for the most common clinical pattern of injury.[9,14,17,19,21] The mice were anesthetized with 16 μL of ketamine and 8 μL of xylazine in 25 μL of phosphate-buffered saline by intraperitoneal injection. For histologic analysis, lacerations were performed bilaterally on the gastrocnemius muscle through 50% of its width and 100% of its thickness at 60% of its length from its insertion. All mice were randomly assigned to 1 of 3 groups: (i) a control group, (ii) a low-dose ARB group, or (iii) a high-dose ARB group. The control group was fed tap water, while the low and high-dose ARB groups were fed commercially available losartan (Cozaar; Merck & Co, West Point,

Pennsylvania) dissolved in tap water at concentrations of 0.05 g/L, and 0.5 g/L, respectively. These doses were calculated based on the average fluid intake of a population of mice with similar demographic backgrounds to those tested in this study, and are well below reported toxicity levels. All animals were caged separately and allowed access to the water or ARB solutions ad libitum from the time of injury to the time of sacrifice. The animals were not pretreated in any way. The average daily fluid intake of each animal was monitored and recorded. Mice were sacrificed at 3 and 5 weeks. The injured muscles were isolated, mounted, and snap-frozen in liquid nitrogen-cooled 2-methylbutane. Samples were serially sectioned at 10 μm with a cryostat for histologic analysis.

Angiotensin Receptors in Skeletal Muscle

Immunohistochemistry was used to evaluate the presence and distribution of angiotensin II receptors in injured and uninjured skeletal muscle. Commercially available antibodies (Abcam Inc, Cambridge, Massachusetts) were used to localize angiotensin II receptors in skeletal muscle. The same sections were also stained with collagen IV antibodies (Biodesign International, Saco, Maine), which stain the extracellular matrix (basal lamina) of muscle fibers, and 4′-6-diamidino-2-phenylindole (DAPI) to delineate nuclear location. A blinded observer performed all analyses.

Muscle Fibrosis

To detect the amount of fibrosis in the area of injury, sections from each limb of each animal were washed in deionized water and stained with a Masson Modified IMEB Trichrome Stain Kit (IMEB Inc, San Marcos, California) according to the manufacturer's specifications. This technique, which stains nuclei black, muscle red, and collagen blue, was previously validated through immunohistochemistry as an accurate technique for evaluating fibrous tissue within skeletal muscle.[6,13,20] Five randomly selected 200× high-powered image fields within the injured area for each limb were obtained using a Nikon Eclipse 800 light microscope (Tokyo, Japan) fitted with a Spot-RT digital camera (Diagnostic Instruments, Sterling Heights, Michigan). Images were analyzed using Northern Eclipse image analysis software (Empix Imaging) to measure the percent area of collagen (blue staining tissue) within the injury zone. Color threshold levels within the software program were set to isolate the blue staining regions and calculate the area of that region that corresponded to the area of fibrosis. This value was expressed as a percentage of the entire cross-sectional area of the muscle section. A blinded observer performed all analyses.

Muscle Regeneration

Muscle sections were stained with hematoxylin and eosin. Muscle regeneration was assessed by counting the number of centronucleated myofibers. Five 200× high-powered fields throughout the injury zone were analyzed from each muscle, and the average number of regenerating myofibers per field was calculated. A blinded observer performed all analyses.

TABLE 1
Varying Concentrations of Angiotensin II With Myoblast (C2C12) and Fibroblast (3T3, PP2) Cell Lines Did Not Affect Proliferation at 24, 48, or 72 Hours[a]

Concentrations, M	24 h	48 h	72 h
C2C12			
0	125	114	160
10^{-8}	145	135	150
10^{-6}	130	140	200
10^{-4}	110	135	140
PP2			
0	25	25.5	40
10^{-8}	30	21.5	65.5
10^{-6}	25	24.5	37.5
10^{-4}	22	24.5	34.5
3T3			
0	102.5	570	1205
10^{-8}	111.5	611.5	1144
10^{-6}	124.25	612.5	1158
10^{-4}	141.875	139.8	112.7

[a]Angiotensin II concentration is expressed in molar amounts, and cell counts are expressed as thousands of cells ($\times 10^3$ cells).

Statistical Methods

Comparisons of muscle fibrotic area and number of regenerating myofibers data were performed by means of a 2-way analysis of variance (ANOVA) and Tukey's post-hoc test using SPSS software, version 16.0 (SPSS Inc, Chicago, Illinois). Immunohistochemical data are presented and compared qualitatively.

RESULTS

In Vitro

Angiotensin II does not affect cell proliferation. Angiotensin II appeared to have no effect on C2C12 (myoblast cell line), NIH 3T3 (fibroblastic cell line), or PP2 (fibroblast cell line) proliferation in vitro compared with controls at 24, 48, or 72 hours of culture (Table 1).

Angiotensin II increases the production of TGFβ-1 in fibroblasts. Fibroblasts isolated from normal skeletal muscle (PP2 cells) appear to up-regulate the expression of TGF-β1 detected by ELISA in the supernatant fluid when cultured with angiotensin II in a dose-independent manner at both 24 and 48 hours. National Institutes of Health 3T3 fibroblasts also appear to respond to the presence of angiotensin II by expressing TGF-β1, as detected by western blotting (data not shown).

In Vivo

Angiotensin II receptors in skeletal muscle. Immunohistochemistry demonstrated the presence of angiotensin II receptors in both normal and injured skeletal muscle. The

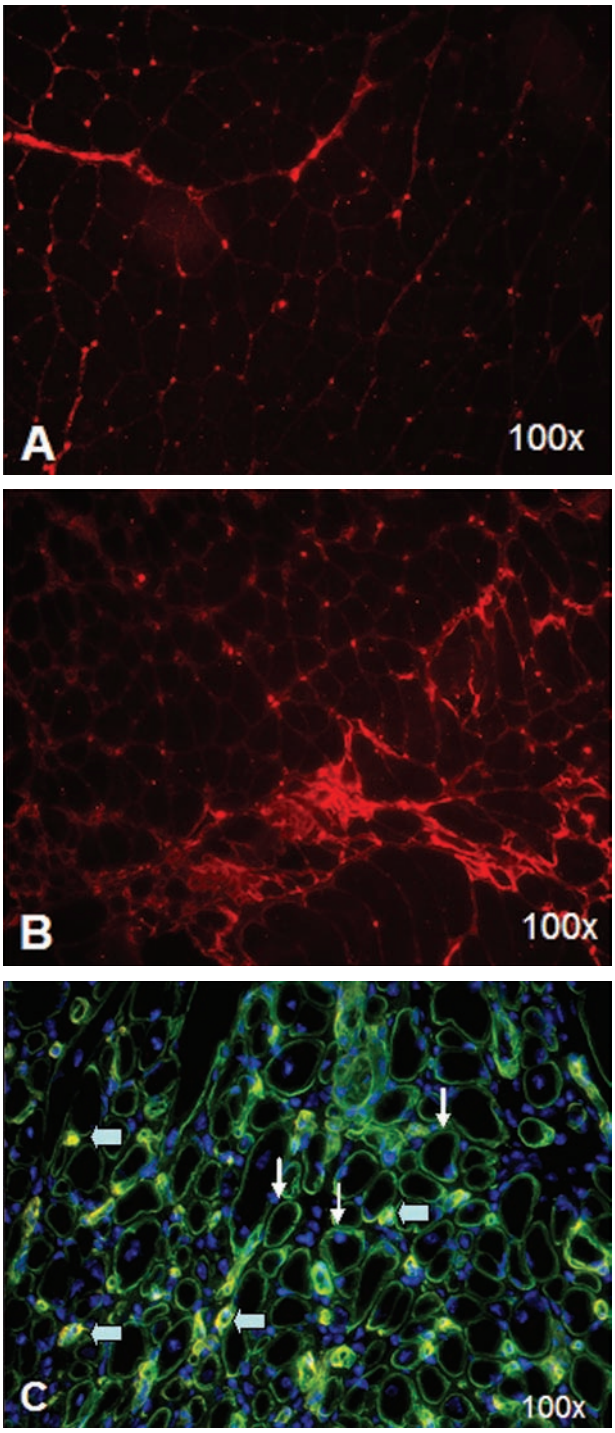


Figure 1. Immunohistochemistry demonstrating increased expression of the angiotensin II receptor (red) in injured (B) versus noninjured (A) skeletal muscle. C, the spatial relationships between collagen IV (green), angiotensin II receptor (red), nuclei (blue), and colocalization (yellow) are shown. The angiotensin II receptor distribution (thick arrows) appears more frequently within the extracellular matrix and sarcolemma of muscle fibers than within the myofibers themselves (thin arrows).

distribution of the receptors in normal muscle appeared to be sparse and evenly distributed along the cell membrane. After injury, the expression of angiotensin II receptors appeared elevated, particularly within the zone of injury. The expression of these receptors appeared to be greater within the cellular structure of the extracellular matrix, rather than on the membrane of the muscle fibers (Figure 1).

Animal Model

All mice used in this experiment were male. No mice demonstrated any ill effects from the treatment regimen, and there were no observable differences in the activity level in any of the groups; no deaths were attributable to the therapeutic intervention. The age of all animals at the beginning of the study ranged from 4 to 6 weeks, and the average weight was 21.4 ± 2.3 g. There was no significant difference in the weight of the animals in the experimental or control groups at any time point. The average dose of ARB received by each animal in the low-dose group and the high-dose group was approximately 0.027 ± 0.015 g/kg/d and 0.298 ± 0.20 g/kg/d, respectively.

Skeletal Muscle Regeneration

At 3 weeks, the average number of regenerating myofibers within the zone of injury (as identified by centrally located nuclei) was 56 ± 14 fibers per 200× high-powered field (n = 4) in the control group, 93 ± 25 (n = 8) in the low-dose group, and 95 ± 32 (n = 8) in the high-dose group (Figure 2). In injured animals treated for 5 weeks, a statistically significant increase in regenerating myofibers was observed. This increase was 45 ± 16 fibers per 200× high-powered field (n = 5) in the control group, 86 ± 19 (n = 8; $P < .01$ vs control group) in the low-dose group, and 103 ± 21 (n = 7; $P < .01$ vs control group) in the high-dose group. At both 3 and 5 weeks, the authors observed a trend toward increasingly greater numbers of regenerating myofibers as both the dose and duration of treatment increased (Figure 2).

Skeletal Muscle Fibrosis

Three weeks after muscle injury, without benefit of treatment, the control group had an area of fibrosis within the zone of injury equal to 34% ± 17% (n = 4). The low-dose treatment group had significantly less fibrosis within the zone of injury (16% ± 9%; n = 8; $P < .05$) when compared with the control group. The high-dose treatment group also had significantly less fibrosis (7% ± 4%; n = 8; $P < .01$ vs control) when compared with the control group. At 5 weeks after injury, the control group, low-dose group, and high-dose group had fibrosis areas of 45% ± 22% (n = 5), 19% ± 9% (n = 8; $P < .01$ vs control), and 14% ± 11% (n = 7; $P < .01$ vs control), respectively, demonstrating a similar trend toward an attenuation of fibrosis mediated by the administration of losartan (Figure 3).

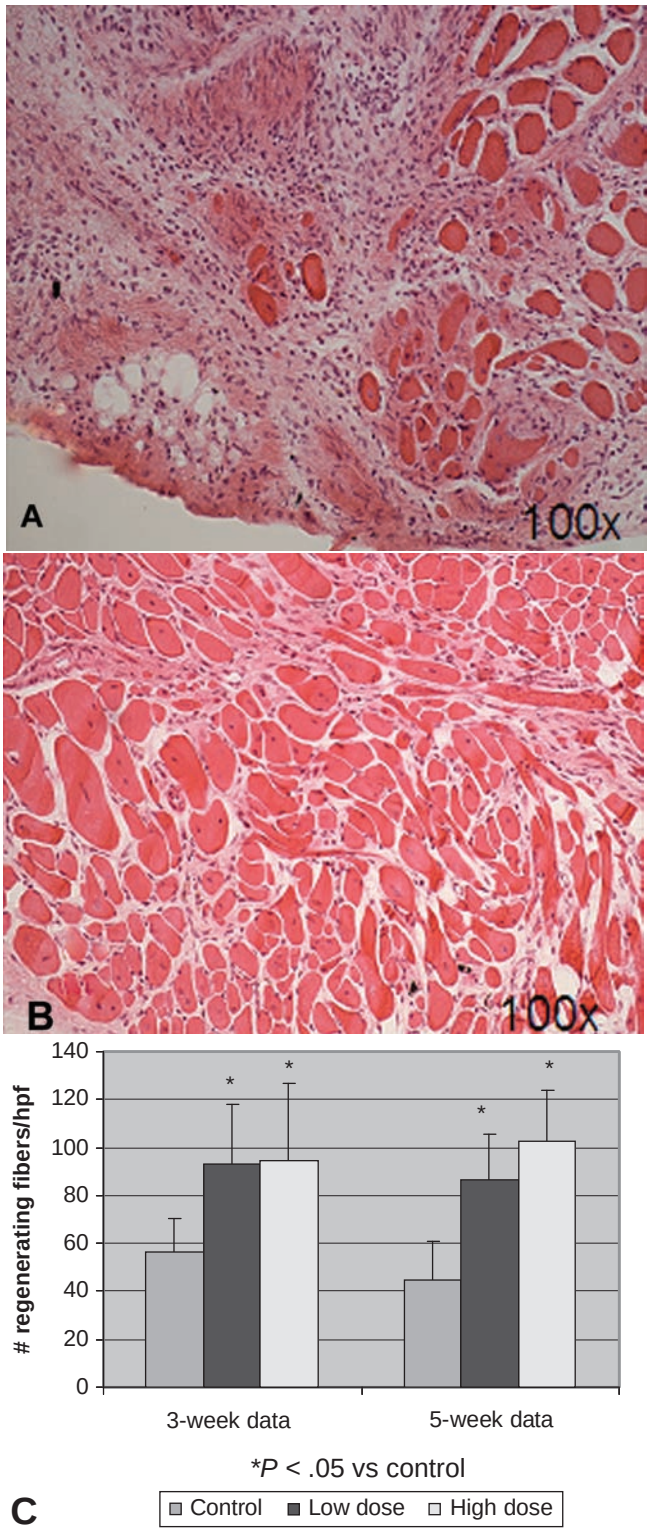


Figure 2. Characteristic hematoxylin-and-eosin–stained sections taken 5 weeks after laceration injury of the gastrocnemius muscle. Note the greater number of regenerating myofibers (recognized by their centrally located nuclei) in the high-dose angiotensin receptor blocker (ARB)–treated group (B) compared with the control group (A). The graph (C) depicts an increase in the number of regenerating myofibers in ARB-treated animals compared with controls. Error bars represent standard deviation. hpf, high-powered field.

DISCUSSION

The impetus for investigating the antifibrotic properties of ARBs in muscle healing arises from prior research on angiotensin II, a known vasoconstrictor and target of many antihypertensive medications, demonstrating its deleterious effects on smooth and myocardial muscle tissues after injurious insults. The role of angiotensin II in muscle fibrosis after injury is well documented in the cardiac literature, in which antagonism of angiotensin II with ARBs is noted to significantly improve cardiac contractility and cardiac output.[15] Moreover, histologic analysis indicates that the mechanism of cardiac improvement is related to decreased fibrosis and enhanced regeneration. The effect of ARBs on skeletal muscle healing, however, remains largely unstudied until recently.

The natural history of skeletal muscle injury is that of residual fibrosis within regenerate tissues that predisposes muscle to reinjury and reduced functionality, thereby constituting a significant clinical burden. After injury, the initial phase of myofiber regeneration wanes over time as it is replaced by fibrous tissue. A reduction of fibrous tissue and the improvement of muscle regeneration correlate with enhanced function.

Several investigations have observed this by treating injured skeletal muscle with antifibrotic agents that directly antagonize TGF-β1, and the digestion of existing fibrous tissue deposits appears to enhance myofiber regeneration.[5,9,13,14,23,24] Research on the pathogenesis of fibrosis further reveals that local levels of TGF-β1 during the fibrotic phase are elevated along the zone of injury[21] as a potential result of angiotensin II.[10]

In a mechanism consistent with these studies, we show through ELISA and western blot assays that fibroblasts increase TGF-β1 production when they are exposed to angiotensin II. We also show that angiotensin II receptors are more densely distributed within the extracellular matrix of the zone of injury, suggesting that angiotensin II receptor upregulation is intimately related to the deposition of fibrous tissue. Our results further demonstrate that inhibiting angiotensin II with an ARB diminishes post-traumatic fibrosis.

We have previously focused our attention on muscle injury models such as ischemia, contusion, strain, and cardiotoxin injection. While skeletal muscle laceration is a less frequently observed injury pattern, its histopathology is nearly identical to the more commonly observed strain injury patterns. Our purpose in choosing the laceration model in this experiment was 2-fold. First, it represents a reproducible model for evaluating skeletal muscle injury, which is a challenge with the more common strain-type injury model because of difficulty in accurately reproducing and delivering the same magnitude of injury between samples. Second, the laceration model establishes a "worst-case scenario" in which myofibers are completely transected. Observations of any treatment effect in this worst-case injury model would likely be of equal or, perhaps, greater benefit in less severe and more commonly encountered problems like strain injuries. In addition, the animals were not pretreated with ARB before injury, as this more closely resembles clinically relevant scenarios in which muscle injuries are treated. While many potential

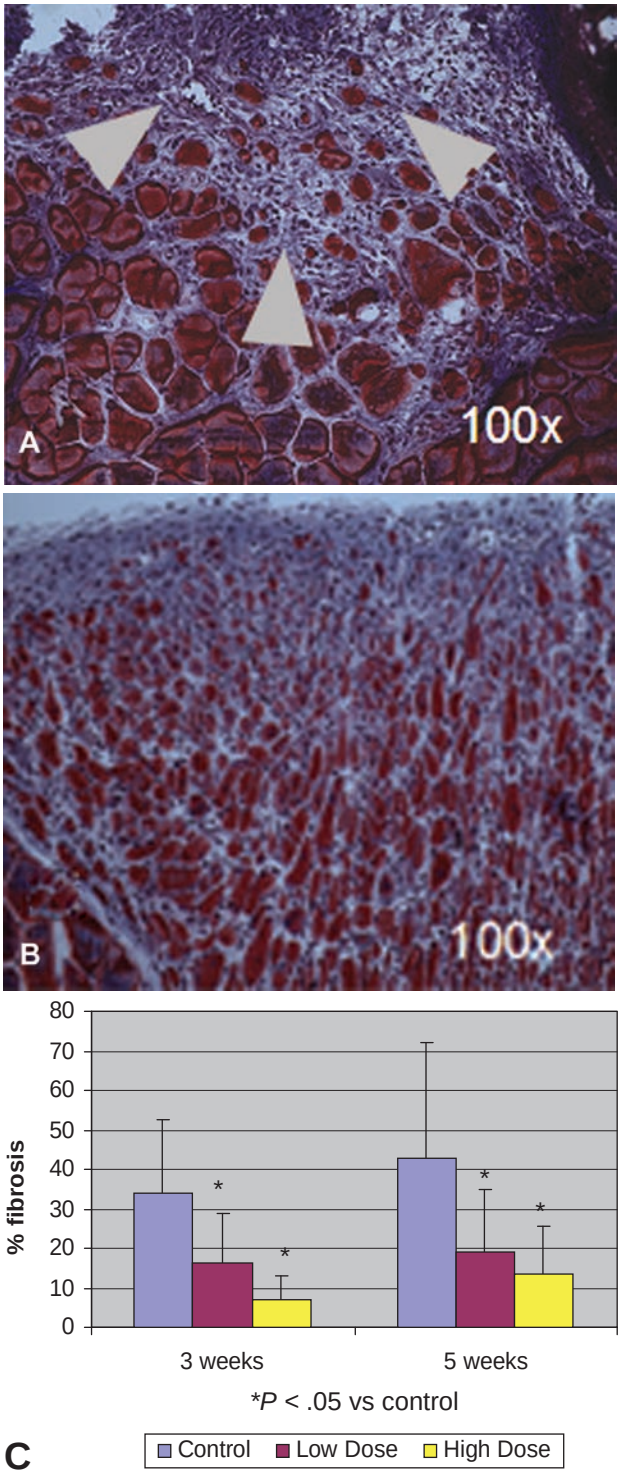


Figure 3. Masson's trichrome (collagen: blue, myofibers: red, nuclei: black) staining of sections taken 5 weeks after laceration injury of the gastrocnemius. Note the dense blue staining fibrous tissue in the control group (A) (arrowheads) compared with the high-dose angiotensin receptor blocker (ARB)–treated group (B). Both pictures are taken at 100× magnification. The graph (C) depicts a decrease in the amount of fibrosis in ARB-treated animals compared with controls. Error bars represent standard deviation.

problems exist with the use of an antihypertensive medication, all doses administered to the animals in this study were below the corresponding human dose equivalent for the antihypertensive effect of losartan. Accordingly, there were no ill effects observed in any of the animals.

Treating skeletal muscle-injured mice with an ARB for extended periods of time reduces the amount of fibrosis and enhances the number of regenerating myofibers in the zone of injury. This occurs in a duration-dependent manner, where medication administered for longer periods of time provided greater regenerative benefit. The observation of a positive regenerative effect after only 5 weeks of treatment, as opposed to other models where treatment was given for up to 9 months, is of notable significance. Prolonged treatment for acute skeletal muscle injuries would seem to be a less attractive and less pragmatic approach.

Similar success in reducing residual fibrosis and enhancing myofiber regeneration, as well as success in improving function, has been observed in animal models, albeit after treatment with potentially harmful compounds. As noted above, we have demonstrated that decorin, suramin, gamma-interferon, and relaxin can all decrease residual fibrosis, enhance myofiber regeneration, and improve function in animal models of skeletal injury.[9,13,14,22,24] However, these compounds are not commonly employed in clinical practice, and several of these are known to have relatively severe side effects that may preclude their use in the treatment of muscle injury.

While the clinical implications of identifying efficacious antifibrotic therapies are particularly important in the field of sports medicine, they apply to an even broader spectrum of orthopaedic patients. Athletes with muscle strains often struggle with lengthy recovery periods in rehabilitation that can limit their duration of play and predispose them to recurrent injuries that oftentimes are more severe.[26] For orthopaedic trauma patients, contusions and lacerations are markedly common, and for all patients undergoing elective and emergent procedures, many surgical incisions are essentially controlled lacerations of skeletal muscle. In each type of patient, the quality of regenerate skeletal muscle and the rate of recovery are, ultimately, compromised and delayed by fibrosis. For some patients, this can result in prolonged immobilization with greater risks for developing a deep venous thrombus or pneumonia. Because the clinical use of angiotensin receptor II inhibitors is not currently approved for orthopaedic surgery, clinical trials are necessary to further determine the effect of this class of medication on skeletal muscle healing, as well as on the functional outcomes, morbidity, and mortality associated with skeletal muscle injury and prolonged rehabilitation.

In light of the possibility that our work may pave the way for clinical trials on the effect of losartan as an antifibrotic therapy to promote optimal skeletal muscle healing, it is important to draw attention to potential adverse effects of angiotensin II receptor antagonists. The side effect profile of ARBs is surprisingly minimal compared with other antihypertensive medications; the only reported dose-related side effect is hypotension, while non-dose–related adverse outcomes include headaches, dizziness,

weakness, and fatigue.[3] This is particularly worth mentioning because these side effects can theoretically affect athletic performance on the field as well as patient performance in a rehabilitation program. Accordingly, clinicians and therapists must be vigilant in monitoring for such effects. While uncommon, there have also been reports of raised liver enzymes, cholestatic hepatitis, and pancreatitis with losartan.[7,8,23] Finally, while ARBs are more well known for their renoprotective effects, any diuretic generally carries the potential for acute renal insufficiency, which can be a particular concern that especially warrants mentioning in settings where patients may be dehydrated from athletic exertion or blood losses in postoperative and trauma settings.

In summary, the use of the ARB losartan, a commercially available, generally well-tolerated, safe, and widely used antihypertensive medication, has been demonstrated in this study to enhance muscle healing by reducing fibrosis and enhancing muscle regeneration in doses below those required for any antihypertensive effect. These findings may represent the most clinically appealing and potentially far-reaching antifibrotic therapy developed so far for otherwise healthy individuals who have sustained acute skeletal muscle injuries.

ACKNOWLEDGMENT

The authors thank David Humiston for editorial assistance. This work was supported by grants to Dr Johnny Huard from the National Institutes of Health (1 R01 AR047973), the William F and Jean W Donaldson Chair at Children's Hospital of Pittsburgh, and the Henry J Mankin Chair at the University of Pittsburgh.

REFERENCES

1. Almekinders LC. Anti-inflammatory treatment of muscular injuries in sports. *Sports Med.* 1993;15(3):139-145.
2. American Academy of Family Physicians. *ICD-9 Codes for Family Medicine 2005-2006*. 2005.
3. Aronson, Jeffrey K. Angiotensin II receptor antagonists. In: JK Aronson, ed. *Meyler's Side Effects of Drugs: The International Encyclopedia of Adverse Drug Reactions and Interactions*. Amsterdam: Elsevier; 2006:223-226.
4. Bedair H, Li Y, Huard J. Angiotensin receptor blockers improve muscle regeneration and decrease fibrosis in injured skeletal muscle. *Trans Orthop Res Soc.* 2005;30:524.
5. Bedair H, Liu T, Kaar J, et al. Matrix metalloproteinase-1 therapy improves muscle healing. *J Appl Physiol.* 2007;102:2338-2345.
6. Best TM, Shehadeh SE, Leverson G, Michel JT, Corr DT, Aeschlimann D. Analysis of changes in mRNA levels of myoblast- and fibroblast-derived gene products in healing skeletal muscle using quantitative reverse transcription-polymerase chain reaction. *J Orthop Res.* 2001; 19(4):565-572.
7. Bosch X. Losartan-induced acute pancreatitis. *Ann Intern Med.* 1997;127:1043–1044.
8. Bosch X. Losartan-induced hepatotoxicity. *JAMA.* 1997;278:1572.
9. Chan YS, Li Y, Foster W, et al. Antifibrotic effects of suramin in injured skeletal muscle after laceration. *J Appl Physiol.* 2003;95(2):771-780.
10. Cohn RD, van Erp C, Habashi JP, et al. Angiotensin II type 1 receptor blockade attenuates TGF-β–induced failure of muscle regeneration in multiple myopathic states. *Nat Med.* 2007;13(2):204-210.
11. Cozaar [package insert]. West Point, PA: Merck & Co Inc; 1995.
12. Folland J, Leach B, Little T, et al. Angiotensin-converting enzyme genotype affects the response of human skeletal muscle to functional overload. *Exp Physiol.* 2000;85(05):575-579.
13. Foster W, Li Y, Usas A, Somogyi G, Huard J. Gamma interferon as an antifibrosis agent in skeletal muscle. *J Orthop Res.* 2003;21(5):798-804.
14. Fukushima K, Badlani N, Usas A, Riano F, Fu F, Huard J. The use of an antifibrosis agent to improve muscle recovery after laceration. *Am J Sports Med.* 2001;29(4):394-402.
15. Gremmler B, Kunert M, Schleiting H, Ulbricht JL. Improvement of cardiac output in patients with severe heart failure by use of ACE-inhibitors combined with the AT1-antagonist eprosartan. *Eur J Heart Fail.* 2000;2(2):183-187.
16. Huard J, Li Y, Fu FH. Muscle injuries and repair: current trends in research. *J Bone Joint Surg Am.* 2002;84(5):822-832.
17. Hurme T, Kalimo H, Lehto M, Jarvinen M. Healing of skeletal muscle injury: an ultrastructural and immunohistochemical study. *Med Sci Sports Exerc.* 1991;23(7):801-810.
18. Jarvinen MJ, Lehto MU. The effects of early mobilization and immobilization on the healing process following muscle injuries. *Sports Med.* 1993;15(2):78-89.
19. Kalimo H, Rantanen J, Jarvinen M. Muscle injuries in sports. *Baillieres Clin Orthop.* 1997;2:1-24.
20. Kherif S, Lafuma C, Dehaupas M, et al. Expression of matrix metalloproteinases 2 and 9 in regenerating skeletal muscle: a study in experimentally injured and mdx muscles. *Dev Biol.* 1999;205(1):158-170.
21. Li Y, Huard J. Differentiation of muscle-derived cells into myofibroblasts in injured skeletal muscle. *Am J Pathol.* 2002;161(3):895-907.
22. Li Y, Negishi S, Sakamoto M, Usas A, Huard J. The use of relaxin improves healing in injured muscle. *Ann N Y Acad Sci.* 2005;1041: 395-397.
23. Lim DS, Lutucuta S, Bachireddy P, et al. Angiotensin II blockade reverses myocardial fibrosis in a transgenic mouse model of human hypertrophic cardiomyopathy. *Circulation.* 2001;103(6):789-791.
24. Negishi S, Li Y, Usas A, Fu FH, Huard J. The effect of relaxin treatment on skeletal muscle injuries. *Am J Sports Med.* 2005;33(12):1816-1824.
25. Onder G, Vedova CD, Pahor M. Effects of ACE inhibitors on skeletal muscle. *Curr Pharm Des.* 2006;12(16):2057-2064.
26. Orchard J, Best T. The management of muscle strain injuries: an early return versus the risk of recurrence. *Clin J Sport Med*. 2002;12(1):3-5.
27. Otsuka M, Takahashi H, Shiratori M, Chiba H, Abe S. Reduction of bleomycin-induced lung fibrosis by candesartan cilexetil, an angiotensin II type 1 receptor antagonist. *Thorax.* 2004;59(1):31-38.
28. Paizis G, Gilbert RE, Cooper ME, et al. Effect of angiotensin II type 1 receptor blockade on experimental hepatic fibrogenesis. *J Hepatol.* 2001;35(3):376-385.
29. Qu-Petersen Z, Deasy B, Jankowski R, et al. Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. *J Cell Biol.* 2002;157(5):851-864.
30. Suga S, Mazzali M, Ray PE, Kang DH, Johnson RJ. Angiotensin II type 1 receptor blockade ameliorates tubulointerstitial injury induced by chronic potassium deficiency. *Kidney Int.* 2002;61(3):951-958.
31. Swedberg K, Kjekshus J. Effects of enalapril on mortality in severe congestive heart failure: results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS). *Am J Cardiol.* 1988;62(2):60A-66A.
32. Woods C, Hawkins RD, Maltby S, et al. The Football Association Medical Research Programme: an audit of injuries in professional football—analysis of hamstring injuries. *Br J Sports Med.* 2004; 38(1):36-41.
33. Worrell TW. Factors associated with hamstring injuries. An approach to treatment and preventative measures. *Sports Med.* 1994;17(5): 338-345.

ORIGINAL ARTICLE

Journal of Cellular Physiology

Interaction Between Macrophages, TGF-β1, and the COX-2 Pathway During the Inflammatory Phase of Skeletal Muscle Healing After Injury

WEI SHEN,[1,3] YONG LI,[2,3] JINHONG ZHU,[1,3] RETO SCHWENDENER,[4] AND JOHNNY HUARD[1,2,3]*

[1]*Department of Bioengineering, University of Pittsburgh, Pittsburgh, Pennsylvania*

[2]*Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania*

[3]*Stem Cell Research Center, Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania*

[4]*Laboratory of Liposome Research, Institute of Molecular Cancer Research, University of Zurich, Zurich, Switzerland*

Inflammation, an important phase of skeletal muscle healing, largely involves macrophages, TGF-β1, and the COX-2 pathway. To improve our understanding of how these molecules interact during all phases of muscle healing, we examined their roles in muscle cells in vitro and in vivo. Initially, we found that depletion of macrophages in muscle tissue led to reduced muscle regeneration. Macrophages may influence healing by inducing the production of TGF-β1 and PGE_2 in different muscle cell types. We then found that the addition of TGF-β1 induced PGE_2 production in muscle cells, an effect probably mediated by COX-2 enzyme. It was also found that TGF-β1 enhanced macrophage infiltration in wild-type mice after muscle injury. However, this effect was not observed in COX-$2^{-/-}$ mice, suggesting that the effect of TGF-β1 on macrophage infiltration is mediated by the COX-2 pathway. Furthermore, we found that PGE_2 can inhibit the expression of TGF-β1. PGE_2 and TGF-β1 may be involved in a negative feedback loop balancing the level of fibrosis formation during skeletal muscle healing. In conclusion, our results suggest a complex regulatory mechanism of skeletal muscle healing. Macrophages, TGF-β1, and the COX-2 pathway products may regulate one another's levels and have profound influence on the whole muscle healing process.

J. Cell. Physiol. 214: 405–412, 2008.

Tissue naturally responds to injury caused by trauma, microorganisms, toxins, or other causes through inflammation. Cytokines including histamine, bradykinin, serotonin, prostaglandins and others are released by damaged tissue and cause fluid leakage from blood vessels. These cytokines also attract white blood cells to infiltrate and migrate to the injury site to digest pathogens and dead/damaged cells, a process called phagocytosis. White blood cells and cytokines are of paramount importance for protection against infection and the invasion of foreign substances. Furthermore, they are involved with the regeneration of damaged tissues (Prisk and Huard, 2003; Tidball, 2005).

Skeletal muscle injury is a common type of injury associated with sports activities, high speed vehicle accidents, and military combat. In injured skeletal muscle, the inflammation response starts rapidly after trauma, and causes symptoms that include muscle pain, swelling, fever, and loss of mobility. To relieve these symptoms, many different non-steroidal anti-inflammatory drugs (NSAIDs) are used to block the cyclooxygenase (COX) enzymes and the products of the COX pathway, the prostaglandins. Early studies reported that NSAIDs had a favorable effect in quickly reducing muscle weakness and functional loss in the short term following muscle injury (Hasson et al., 1993; Obremsky et al., 1994; Mishra et al., 1995; Dudley et al., 1997). However, some studies reported either no effect or a detrimental effect on long term muscle strength recovery after prolonged NSAIDs treatment (Mishra et al., 1995; Almekinders, 1999). Recently, studies using COX-2 specific inhibitors also showed that the COX-2 pathway, which is mostly induced in pathological situations, is important in promoting muscle regeneration and reducing fibrosis formation (Bondesen et al., 2004; Shen et al., 2005). Furthermore, it is suggested that the prostaglandins, although the cause of uncomfortable symptoms, are actually beneficial in promoting skeletal muscle healing (Horsley and Pavlath, 2003; Pavlath and Horsley, 2003; Prisk and Huard, 2003).

Interestingly, it has been suggested that two other components of inflammation, macrophages and transforming growth factor-beta1 (TGF-β1), are related to the COX-2 pathway. Macrophages are an important source of COX-2 enzymes and prostaglandins during the inflammation phase

Contract grant sponsor: National Institutes of Health;
Contract grant number: 1R01 AR 47973-01.
Contract grant sponsor: Department of Defense;
Contract grant number: W81XWH-06-1-0406.
Contract grant sponsor: Hirtzel Foundation.
William F. and Jean W. Donaldson Chair at Children's Hospital of Pittsburgh.
Henry J. Mankin Endowed Chair for Orthopaedic Research at the University of Pittsburgh.
Contract grant sponsor: National Center for Research Resources, National Institutes of Health, Research Facility Improvement Program;
Contract grant number: C06 RR-14489.

*Correspondence to: Johnny Huard, Stem Cell Research Center, Children's Hospital of Pittsburgh, 4100 Rangos Research Center, 3460 Fifth Ave, Pittsburgh, PA 15213-2583.
E-mail: jhuard@pitt.edu

Received 10 November 2006; Accepted 8 June 2007

DOI: 10.1002/jcp.21212

(Graf et al., 1999; Lee et al., 2002). And, by some reports, the inhibition of the COX-2 pathway reduced the infiltration of macrophages to the injury site (Bondesen et al., 2004; Shen et al., 2005). TGF-β1, well known for its fibrotic effect, was also shown to be important in the inflammation phase as a result of its connection to the COX-2 pathway. TGF-β1 may increase the production of prostaglandin E_2 (PGE_2) through COX-2, and PGE_2 may inhibit fibroblast proliferation and collagen production to balance the fibrotic effect of TGF-β1 (Goldstein and Polgar, 1982; McAnulty et al., 1997; Keerthisingam et al., 2001). Furthermore, macrophages and TGF-β1 may also be connected to one another, as several studies have shown that macrophages may be an important source of TGF-β1 (Khalil et al., 1989, 1993; Wolff et al., 2004). However, little evidence of these interactions has been demonstrated in muscle cells during skeletal muscle injury.

To further the understanding of the healing mechanism of skeletal muscle injury and the role of inflammation in the muscle healing process, we examined the relationship between the COX-2 pathway, macrophages, and TGF-β1 in skeletal muscle inflammation. We found that these important components form a complex network wherein they appear to regulate each other. These interactions may not only modulate muscle inflammation, but also influence the regeneration and fibrosis phases of skeletal muscle healing.

Materials and Methods

Cell isolation and culturing

Myogenic precursor cells (MPCs) were isolated via a previously described preplate technique (Rando and Blau, 1994; Qu et al., 1998). Briefly, gastrocnemius muscles (GMs) were removed from 4-week-old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), and minced with scissors. The minced tissues were enzymatically digested by sequential exposure to collagenase, dispase, and trypsin. The muscle cell extracts were then plated on collagen-coated flasks, and different populations were isolated by re-plating the extracts after different time intervals. The late plated population is usually composed of MPCs that have high myogenic potential when induced by low serum culture medium (Qu et al., 1998). Exudate macrophages were isolated according to the technique described before (Robertson et al., 1993). Briefly, mice were injected intraperitoneally with 30 ml thioglycollate broth (BD Biosciences, San Jose, CA). Three days later, the peritoneal macrophages were harvested by centrifugation. Macrophages and MPCs were used for in vitro experiments along with two other cell lines, NIH 3T3 fibroblasts and C2C12 myoblasts. These three types of cells were maintained in proliferation medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10% horse serum (HS), and 0.5% chicken embryo extract).

ELISA assay

A low serum-containing medium (DMEM supplemented with 1% FBS and 1% HS) was used to culture cells in the experiments of growth factor and cytokine expression. The media from the TGF-β1 (5 ng/ml, Sigma, St. Louis, MO) and PGE_2 (0, 100, 1,000, or 10,000 ng/ml) treatments experiment were collected and kept at −80°C pending enzyme-linked immunosorbent assay (ELISA). The assay was performed as suggested by the manufacturer's protocols (DE0100 PGE_2 ELISA kit, DE1150 $PGF_{2\alpha}$ ELISA kit, R & D Systems, Inc., Minneapolis, MN).

Animal model

The Animal Research and Care Committee at the authors' institution approved all experimental protocols for this study (Protocol 29/04). Twenty-four C57BL/6J mice (males, 6 weeks of age, Jackson Laboratories), 4 COX-2 knock-out mice (males, 6 weeks of age, Taconic farm), and their wild-type controls (males, 6 weeks of age, Taconic farm) were used for in vivo experiments. The GMs of the mice were injected with cardiotoxin (CTX, c3987, Sigma), a snake venom, to create a muscle injury. Briefly, the mice were anesthetized by intraperitoneal injection of 0.03 ml ketamine (100 mg/ml) and 0.02 ml xylazine (20 mg/ml). Ten microliters of diluted CTX (or 9μl CTX + 1μl TGF-β1) was injected in the middle mass of each GM. The mice were sacrificed at different time points after injection (1, 3, 5, or 14 days). The GMs were harvested from both legs for either flow cytometry or histological analysis. For the latter purpose, the GMs were fresh-frozen in 2-methylbutane pre-cooled by liquid nitrogen, and stored at −80°C pending cryosection.

Macrophage depletion

Clodronate liposomes, were prepared as described previously (Seiler et al., 1997; Tyner et al., 2005). The liposomes act as carriers for clodronate, which is toxic to phagocytic cells. Two days before muscle injury, 1 mg of clodronate liposomes (20 mg/ml) was injected intraperitoneally into C57/BL 6J mice to deplete macrophages. Mice injected with empty liposomes were used as controls. Flow cytometry using macrophage marker antibodies (F4/80 and CD-11b) was used to verify the extent of macrophage depletion.

Hematoxylin and eosin (H & E) staining

The cryosections were fixed in 1% glutaraldehyde for 1 min, and then immersed in hematoxylin for 30 sec. After washing with alcohol acid and ammonia water, they were immersed in eosin for 15 sec. After each step, sections were rinsed with distilled water. The sections were then dehydrated by treatment with alcohols of increasing concentrations (70%, 80%, 95%, and 100%). Following this, the sections were treated with xylene and covered with glass slips. Slides were analyzed on a bright field microscope (NIKON Eclipse E800, Nikon, Tokyo, Japan). Northern Eclipse software (Empix Imaging, Cheektowaga, NY) was used to measure the minor axis diameters of centronucleated regenerating myofibers (i.e., the smallest diameter of a myofiber across the central nucleus; 200× magnification; 200 random myofibers obtained from four samples/group).

Western blot

After washing with PBS, Laemmli sample buffer (BioRad, 161-0737, Hercules, CA) was applied to the surface of culture dishes to collect proteins from live cells. For muscle tissue samples, serial cryosections of 10 μm thickness were collected in Eppendorf tubes and treated with T-PER tissue protein extraction agent (78510, Pierce, Rockford, IL) and mixed with Laemmli sample buffer (BioRad, 161-0737). Protein samples were boiled for 5 min, separated on 10% SDS–polyacrylamide electrophoresis gels, and transferred to nitrocellulose membranes. Mouse anti-COX-2 (160112, Cayman, Ann Arbor, MI) and anti-TGF-β1 antibodies (555052, BD Biosciences Pharmingen, San Diego, CA) were applied as primary antibodies, and mouse anti-β-actin (Sigma; 1:8,000) was used for protein quantification. The horseradish peroxidase-conjugated secondary antibodies (Pierce) were diluted to 1:5,000 (v/v). Blots were developed by using SuperSignal West Pico Chemiluminescent substrate (Pierce), and positive bands were visualized on X-ray film. All results were analyzed by Northern Eclipse software (Empix Imaging).

Flow cytometry

The GMs from non-TGF-β1-treated (10 μl cardiotoxin injection) and TGF-β1-treated groups (9 μl cardiotoxin plus 1 μl of 5 ng/ml TGF-β1 injection) were surgically removed before injury, and at 1, 2, 3, and 5 days after injury for serial evaluation. Collagenase, dispase, and trypsin were used to digest the tissue matrix and

isolate the cells. Debris was removed via filtration with 70 μm filters.

Cells were treated with 10% mouse serum (Sigma) to block non-specific binding sites. Primary rat anti-CD-11b (conjugated with FITC, R & D Systems, Inc.) and rat anti-F4/80 (conjugated with APC, BD Biosciences, San Jose, CA) antibodies were used in combination to identify neutrophil and macrophage populations. We added 7-amino-actinomycin D (7-AAD; BD Biosciences Pharmingen, San Diego, CA) to exclude non-viable cells from the analysis. Samples were then analyzed via fluorescence activated cell sorting (FACS) Caliber flow cytometer (BD Biosciences) and CellQuest software (BD Biosciences).

Statistics

A Chi-squared analysis was used to analyze the percent differences between the number of macrophages identified by flow cytometry and western blotting results. Comparisons between two groups were made by the use of an unpaired Student's *t*-test. All other data were analyzed by one-way ANOVA statistical analysis. Post-hoc multiple comparison tests were performed to determine which means differed. A value of $P < 0.05$ was considered statistically significant. Error bars on figures represent the standard deviation.

Results

Macrophage depletion and muscle regeneration

Liposome clodronate was injected intraperitoneally 2 days prior to muscle injury to deplete the macrophage populations in mice. We used flow cytometry to quantify the number of infiltrating macrophages after injury. This macrophage infiltration peaked 2 days post-injury, and decreased quickly after 5 days. Liposome clodronate injection significantly reduced the number of infiltrating macrophages after muscle injury at all time points observed when compared to the empty liposome control group. At 1d, 2d, 3d, and 5d post-injury, liposome clodronate injection decreased macrophage infiltration by 73.2%, 80.2%, 77.4%, and 64.2%, respectively ($P < 0.05$, Fig. 1A). As shown on the flow cytometry graph, the macrophage population, which is shown in the S2 quadrant in the empty liposome control mice, was significantly reduced in the clodronate liposome-injected mice (Fig. 1B).

Clodronate liposome-treated mice exhibited reduced muscle regeneration at both 14 and 28 days after injury. The size of regenerating myofibers in liposome clodronate-treated mice was significantly smaller than those observed in the empty liposome control group ($P < 0.05$, Fig. 1C–E). However, at 7 days post-injury, there was no significant difference between these two groups.

Macrophages depletion decreases the expression of TGF-β1

Injured GMs were isolated from empty liposome control and clodronate liposome-injected mice. We examined the TGF-β1 expression level in injured muscle tissue by using Western blot analysis. Our results indicated that, compared to the empty liposome control group, TGF-β1 expression was significantly decreased 3 and 5 days after injury in the clodronate liposome-injected group, which had its macrophages depleted before the creation of muscle injury ($P < 0.05$, Fig. 2A,B). These results suggest that macrophages play an important role in inducing the expression of TGF-β1 during the inflammatory phase of skeletal muscle after injury.

TGF-β1 induces the production of COX-2 enzyme and prostaglandins

To examine the effect of TGF-β1 on the COX-2 pathway, we cultured MPCs and treated them with TGF-β1. We analyzed the COX-2 enzyme level from MPCs treated with TGF-β1 and the non-treated control cells for 4 days. Western blot analysis showed that COX-2 production was significantly higher in the MPCs treated by TGF-β1 compared to non-treated control cells ($P < 0.05$, Fig. 3A,B). We further tested the expression of PGE_2 from different muscle cells, including MPCs, NIH 3T3 fibroblasts, and C2C12 myoblasts, after they were treated with TGF-β1 for 4 days. We found that TGF-β1 treatment increased the expression of PGE_2 significantly in all cell types, when compared to non-treated control cells ($P < 0.05$, Fig. 3C).

To further validate the relationship between TGF-β1 and the COX-2 pathway, we used wild-type MPCs as a model system. In this experiment, NS-398 (a COX-2 specific inhibitor) was added to the cell culture to block the COX-2 enzyme activity. This additional treatment ablated the increased PGE_2 expression that was induced by adding TGF-β1 into the cell culture ($P < 0.05$, Fig. 3D). In addition, COX-$2^{-/-}$ cells had very low levels of PGE_2 expression, even if TGF-β1 was added. This finding suggests that TGF-β1 regulates the production of COX-2 pathway products, and that the enhanced expression of PGE_2 may be mediated by COX-2.

TGF-β1 increases the infiltration of macrophages through the COX-2 pathway

To examine the effect of TGF-β1 on macrophage infiltration, we created CTX injury (with or without TGF-β1 treatment) on the GMs of wild-type mice. We found that, after TGF-β1 treatment, the number of infiltrating macrophages was significantly increased compared with the CTX-only group at days 1, 2, and 3 after muscle injury ($P < 0.05$, Fig. 4A). The same experiment was conducted on COX-$2^{-/-}$ mice. Interestingly, we found that the number of infiltrating macrophages actually decreased with the addition of TGF-β1, instead of being increased 3 days after injury ($P < 0.05$, Fig. 4B). This further validates the suggestion that the effect of TGF-β1 on macrophage infiltration is mediated at least in part by the COX-2 pathway products.

PGE_2 decreased the expression of TGF-β1

To examine the influence of PGE_2 on fibrosis formation, we chose to examine the effect of PGE_2 treatment on the expression of TGF-β1, which is known for its fibrotic effect especially in skeletal muscle (Li et al., 2004). Three different muscle cell types, MPCs, NIH 3T3 fibroblasts, and C2C12 myoblasts, were tested for TGF-β1 expression following treatment with PGE_2 for 4 days. We found that PGE_2 treatment decreased the expression of TGF-β1 significantly in all cell types tested, when compared to non-treated control cells ($P < 0.05$, Fig. 5). This result suggests that PGE_2 may be able to decrease fibrosis formation after muscle injury by decreasing the expression of fibrotic growth factor TGF-β1.

Discussion

Inflammation is an important phase of skeletal muscle healing. Macrophages, TGF-β1, and the COX-2 pathway are the integral components of this phase. In order to further the understanding of muscle healing and improve the health care of muscle injury patients, we must investigate the importance of these components and elucidate their interaction in muscle healing regulation. In this study, we examined their roles and relationships during the inflammation phase and postulated a possible regulatory mechanism (Fig. 6).

Many researchers suggest that macrophages are important for muscle regeneration (Cantini et al., 2002; Camargo et al., 2003; Chazaud et al., 2003; Tidball, 2005). To create a "Loss of Macrophages Function" condition in skeletal muscle healing, especially in the inflammation phase, we used the clodronate liposome injection technique (Seiler et al., 1997; Tyner et al.,

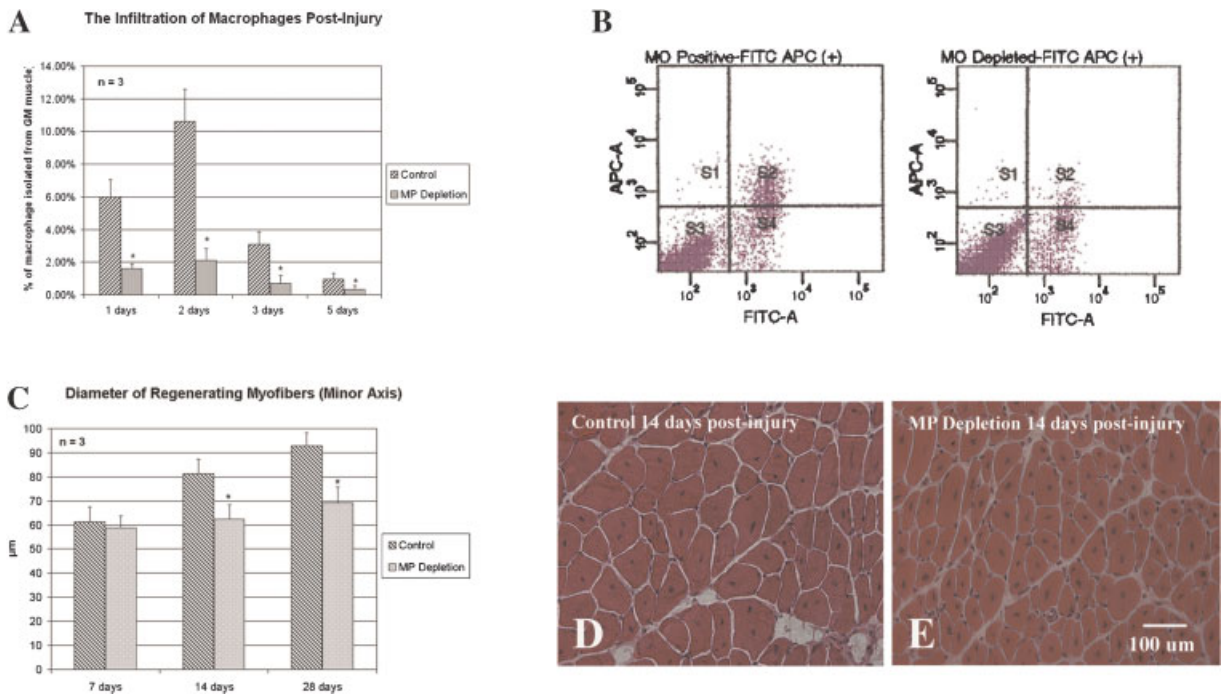


Fig. 1. Injection of clodronate liposome depleted macrophages in injured GM significantly after injury. At 1d, 2d, 3d, and 5d post-injury, liposome clodronate injection decreased macrophage infiltration by 73.2%, 80.2%, 77.4%, and 64.2%, respectively ($P < 0.05$, A). Flow cytometry results showed that the macrophage population, which was shown in the S2 quadrant in non-treated mice at 48 h after injury (B, left), was significantly reduced in clodronate liposome-treated mice (B, right). The X-axis represents the CD-11b cell surface marker; and the Y-axis represents the F4/80 cell surface marker. Compared with empty liposome-control group, clodronate liposome-injected mice exhibited significantly reduced sizes of regenerating myofibers 14 and 28 days post-injury ($P < 0.05$, C). Sample H & E stained GM sections from empty liposome and clodronate liposome-injected groups 14 days post-injury are shown in (D,E). The asterisks indicate a statistically significant difference ($P < 0.05$) between the marked groups and the control group. The error bars in the graph represent the standard deviation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

2005) to deplete the macrophage populations in mice. With the injection of clodronate liposomes, most of the macrophages were depleted and only a few remaining cells infiltrated into muscle tissue during the inflammation phase. We found that, although regeneration of injured muscle did occur, the macrophage depletion group had significantly smaller myofibers than the non-treated group, which is indicative of a delay in muscle regeneration. This suggests that macrophages participate in the muscle healing process, and may play a beneficial role in the growth of regenerating myofibers.

One of the possible mechanisms by which macrophages contribute to the healing process is by inducing the proliferation and differentiation of satellite cells by secreting growth factors and cytokines (Cantini et al., 1994, 1995; Lescaudron et al., 1999; Merly et al., 1999; Chazaud et al., 2003). The factors secreted by macrophages exert their effects not only on specialized satellite cells during muscle regeneration, but have a broader mitotic activity on all myogenic cells (Cantini and Carraro, 1995). In this study, we found that macrophage-depleted mice had significantly lower TGF-β1 levels in their injured GM muscles compared to those from non-treated control mice. This finding suggests that macrophages play a role in modulating the level of these growth factors including TGF-β1 during the healing process of skeletal muscle. However, the exact role of macrophages and their interaction with other muscle cells warrants further investigation. Studies need to be performed to determine the cellular source of growth factors in the muscle inflammation process, and whether direct contact or humeral regulation is necessary to induce the expression of these growth factors.

Fibrosis is a complex biological process that is usually seen in severe muscle injury. Fibroblasts are activated to proliferate and produce abnormal amounts of extracellular matrix (ECM). Damaged skeletal muscle tissue is replaced by the deposition of overproduced ECM (i.e., fibrosis/scar) tissue, instead of regenerating myofibers. TGF-β1 is one of the most potent fibrotic stimuli (Li et al., 2004). It is an inducer of ECM protein synthesis and fibroblast proliferation (Kahari et al., 1991; Taipale et al., 1994), and it has been implicated in the fibrogenesis of various tissues (Border and Noble, 1994). However, the role of TGF-β1 in skeletal muscle healing is not limited to fibrosis. Previous studies have indicated that TGF-β1 may be an immunosuppressive molecule because its elimination or its downstream Smads signaling cascade disruption leads to severe inflammatory disease (Kulkarni and Karlsson, 1997; Yang et al., 1999; Nakao et al., 2000; Monteleone et al., 2004a). On the other hand, it has been suggested by some authors that TGF-β1 is able to increase PGE_2 expression in other tissues (Fawthrop et al., 1997; McAnulty et al., 1997; Keerthisingam et al., 2001; Han et al., 2004). Since it has been suggested that the COX-2 pathway products including PGE_2 and $PGF_{2\alpha}$ are important inflammatory mediators that induce regeneration in skeletal muscle healing (Bondesen et al., 2004; Shen et al., 2005), it is important to explore their relationship with TGF-β1. In our in vitro studies, we found that the addition of TGF-β1 significantly increased the production/expression of both COX-2 enzyme and PGE_2 in muscle cells. By blocking COX-2, TGF-β1's effect on PGE_2 expression was ablated. Furthermore, the effect of TGF-β1 was not observed in COX-$2^{-/-}$ cells. These results indicate that TGF-β1 is not only a fibrotic inducer,

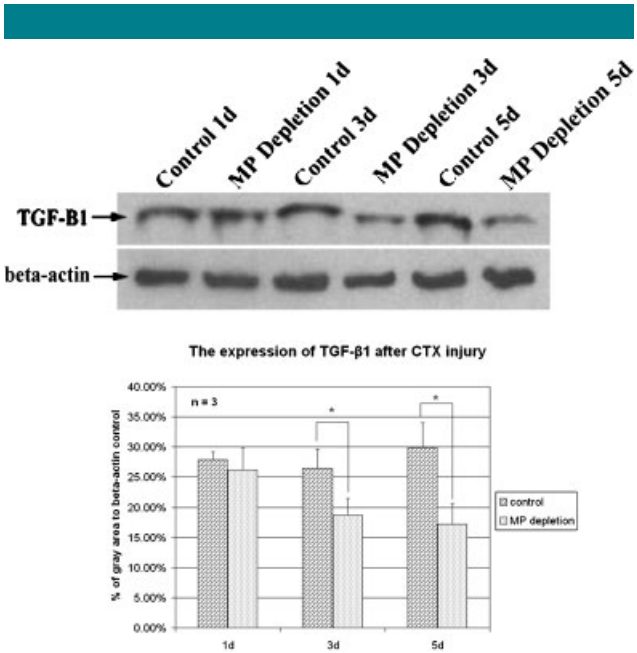


Fig. 2. The in vivo expression level of TGF-β1 was significantly lower in the clodronate liposome-injected mice compared to the empty liposome control mice 3 and 5 days after injury ($P < 0.05$). A representative western blot result is shown at the top. The asterisks indicate a significant difference ($P < 0.05$) between the compared groups. The error bars in the graph represent the standard deviation.

but is also an inflammatory modulator in muscle injury. In fact, TGF-β1 may enhance the inflammatory response by enhancing the COX-2 pathway, especially the production of PGE_2. This may represent an important finding because it indicates that TGF-β1 may both up- and downregulate inflammation through different pathways.

It has been shown that TGF-β1 interferes with the inflammation phase in various ways (Fiocchi, 2001; Warshamana et al., 2002; Monteleone et al., 2004a,b; Wang et al., 2005). Mostly, TGF-β1 was thought to inhibit inflammation because TGF-β1 is a negative regulator of NF-κB activation (Haller et al., 2003). Smad7 maintains high NF-κB activity during inflammation by blocking TGF-β1 signaling (Monteleone et al., 2004a,b; Wang et al., 2005). To elucidate whether TGF-β1 modulates inflammation by other means, macrophage infiltration for example, we injected TGF-β1 together with CTX. The TGF-β1 addition significantly increased the infiltration of macrophages into injured skeletal muscle. This suggests that TGF-β1 interferes with the inflammation phase of muscle healing by increasing the number of infiltrating macrophages. However, in COX-$2^{-/-}$ mice, the number of infiltrating macrophages was decreased with the addition of TGF-β1 to the cardiotoxin injection. This finding suggests that the COX-2 pathway mediates this TGF-β1-induced macrophage infiltration. As prostaglandins are known to cause localized vasodilation, it is reasonable to postulate that they may be at least partly responsible for the chemotaxis of macrophages. In future experiments, prostaglandin's chemotactic property as well as TGF-β1's regulation of NF-κB should be more deeply investigated. If it could be shown that TGF-β1 also has a negative impact on inflammation through the downregulation of NF-κB, it would be intriguing to discover which pathway is dominant in muscle injury, and determine why TGF-β1 would have contradictory effects. Based on the findings that macrophages increased the expression of TGF-β1, and TGF-β1 increased the infiltration of macrophages, which is mediated by the COX-2 pathway, they may form a positive feedback loop to further enhance the number of infiltrating macrophages in the injured muscle (Fig. 6).

A previous study of liver fibrosis showed that PGE_2 inhibited TGF-β1-mediated induction of collagen alpha I production in

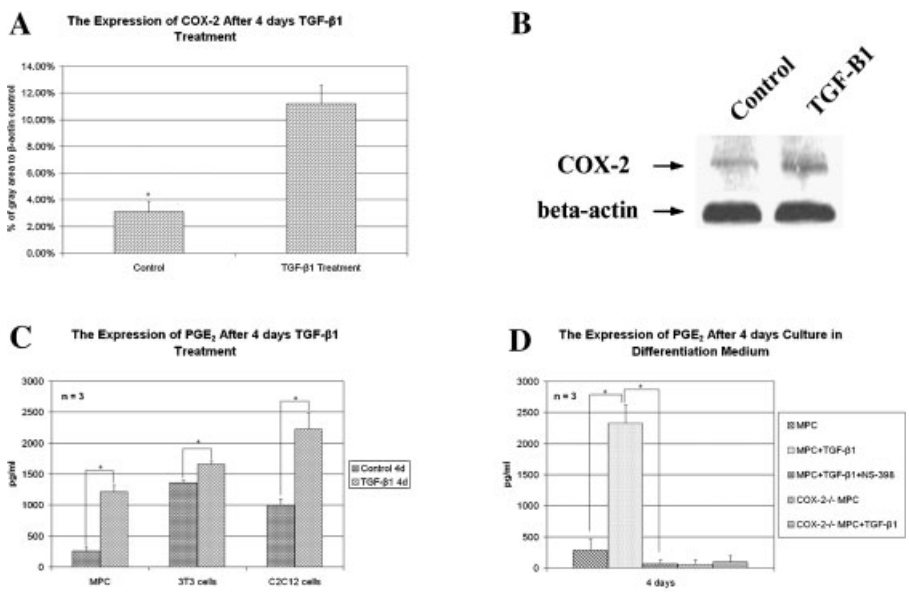


Fig. 3. MPCs that were treated with TGF-β1 for 4 days had significantly higher production of COX-2 enzyme than the non-treated control cells ($P < 0.05$, A). A representative Western blot result is shown in (B). Muscle cells that were treated by TGF-β1 for 4 days had significantly higher expression levels of PGE_2 compared with the non-treated control cells ($P < 0.05$, C). NS-398 can significantly reduce the increased PGE_2 expression that was induced by TGF-β1 ($P < 0.05$, D). COX-$2^{-/-}$ MPC cells, both treated and non-treated with TGF-β1, expressed PGE_2 at very low levels. The asterisks indicate a significant difference ($P < 0.05$) between the compared groups. The error bars in the graph represent the standard deviation.

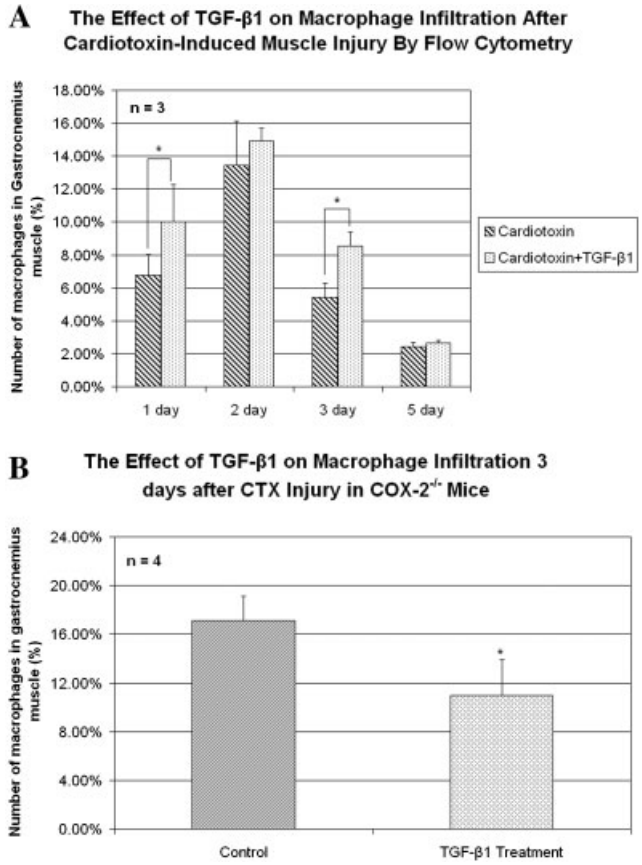


Fig. 4. Flow cytometry results showed that CTX injection can create injury in skeletal muscle and induce the infiltration of macrophages. The addition of TGF-β1 significantly increased the number of infiltrating macrophages at 1 and 3 days after CTX-induced muscle injury ($P < 0.05$, A). In contrast, CTX-injured COX-2$^{-/-}$ mice showed significantly decreased numbers of infiltrating macrophages 3 days post-injury ($P < 0.05$, B). The asterisks indicate a significant difference ($P < 0.05$) between the compared groups. The error bars in the graph represent the standard deviation.

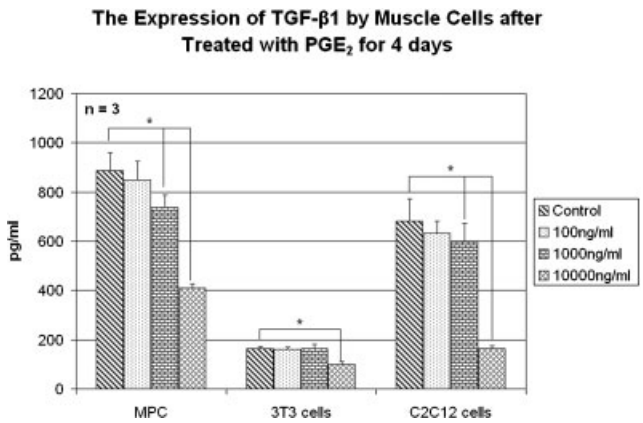


Fig. 5. At concentrations of 1,000 ng/ml and 10,000 ng/ml, PGE_2 decreased the expression of TGF-β1 significantly in both MPCs and C2C12 myoblasts. However, PGE_2 was able to decrease the expression of TGF-β1 in NIH 3T3 fibroblasts only at a concentration of 10,000 ng/ml ($P < 0.05$). The asterisks indicate a significant difference ($P < 0.05$) between the compared groups. The error bars in the graph represent the standard deviation.

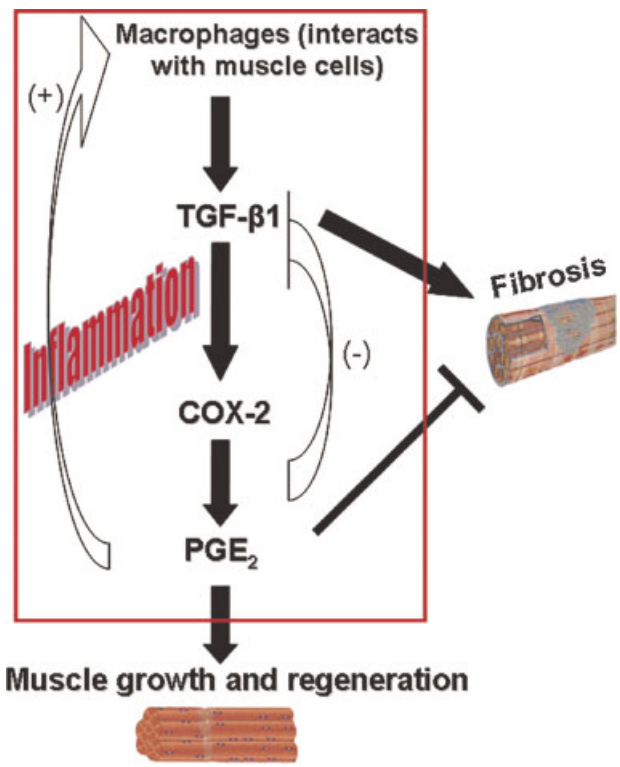


Fig. 6. The proposed mechanism of muscle inflammation is shown. Macrophages interact with muscle cells to increase the level of TGF-β1 in muscle inflammation. TGF-β1 increases the level of prostaglandins, which mediates muscle regeneration and the further infiltration of macrophages, forming a positive feedback loop. In turn, PGE_2 decreases the expression of TGF-β1 and forms a negative feedback loop to downregulate the fibrosis formation. Macrophages, muscle cells, TGF-β1, and COX-2 pathway form a complex network to regulate each other, and thus modulate the following regeneration and fibrosis formation during the healing process after injury. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

hepatic cells (Hui et al., 2004). This finding suggests that PGE_2 and TGF-β1 are able to regulate one another's levels by forming a negative feedback loop resulting in homeostasis of fibrosis formation. To test this phenomenon in skeletal muscle, we treated different muscle cell types with PGE_2 and analyzed their TGF-β1 expression. What we found was that PGE_2 treatment decreased the expression of TGF-β1 significantly in all muscle cell types, when compared to non-treated control cells. On the other hand, our previous study showed that by using NS-398 to block COX-2, and thus the expression of PGE_2, the expression of TGF-β1 was increased in injured muscle tissue (Shen et al., 2005). These results indicate that the TGF-β1 level was probably regulated by PGE_2. The application of NS-398 probably inhibited PGE_2 expression and led to a high level of TGF-β1, and subsequent fibrosis formation in injured muscle. Our findings are consistent with the results from a previous liver fibrosis study (Hui et al., 2004), and further suggests the existence of a negative feedback loop between TGF-β1 and PGE_2 in skeletal muscle (Fig. 6).

PGE_2 is also a potent inhibitor of fibroblast proliferation (Goldstein and Polgar, 1982; Durant et al., 1989) and collagen

synthesis (Goldstein and Polgar, 1982; Saltzman et al., 1982). This suggests that PGE_2 may play an important role in minimizing ECM production. This is especially important in an environment favoring fibrosis formation, such as is found within the inflammation of damaged liver. Interestingly, our preliminary data showed that different concentrations of PGE_2 may affect the proliferation of muscle cells differently (data not shown). Thus, the next step in our study would be to assess the level of PGE_2 in injured skeletal muscle, which should give further insight into the effect of PGE_2 on muscle cell proliferation. It is also important to investigate the role of other prostaglandins, such as $PGF_{2\alpha}$, PGE_2, and 15-PG dehydrogenase in the future, because other prostaglandins may also play a role in the muscle healing process, and 15-PG dehydrogenase may have a significant effect on prostaglandin levels.

In summary, our study demonstrates that the COX-2 pathway, macrophages, and TGF-β1 are important components of the inflammation phase of injured skeletal muscle. Inflammation affects the overall healing of skeletal muscle through these cellular and molecular components. In addition, we found that these components may modulate the production of each other and form a complex co-regulatory mechanism. As we begin to comprehend the fact that simply blocking inflammation by using NSAIDs has a potential downside on muscle healing, more studies are warranted to improve the quality of healthcare in patients afflicted with skeletal muscle injuries.

Acknowledgments

We thank Alison Logar for her tremendous help with flow cytometry analysis, Maria Branca and Ying Tang for their technical support, and Drs. Partha Roy, Bruno Péault, and Patricia Hebda for their helpful discussion. This work was supported by funding from the National Institutes of Health (1R01 AR 47973-01 to JH), the Department of Defense (W81XWH-06-1-0406), the Orris C. Hirtzel and Beatrice Dewey Hirtzel Memorial Foundation, the William F. and Jean W. Donaldson Chair at Children's Hospital of Pittsburgh, and the Henry J. Mankin Endowed Chair for Orthopaedic Research at the University of Pittsburgh. This investigation was conducted in a facility constructed with support from Research Facility Improvement Program Grant Number C06 RR-14489 from the National Center for Research Resources, National Institutes of Health.

Literature Cited

Almekinders LC. 1999. Anti-inflammatory treatment of muscular injuries in sport. An update of recent studies. Sports Med 28:383–388.

Bondesen BA, Mills ST, Kegley KM, Pavlath GK. 2004. The COX-2 pathway is essential during early stages of skeletal muscle regeneration. Am J Physiol Cell Physiol 287:C475–C483.

Border WA, Noble NA. 1994. Transforming growth factor beta in tissue fibrosis. N Engl J Med 331:1286–1292.

Camargo FD, Green R, Capetenaki Y, Jackson KA, Goodell MA. 2003. Single hematopoietic stem cells generate skeletal muscle through myeloid intermediates. Nat Med 9:1520–1527.

Cantini M, Carraro U. 1995. Macrophage-released factor stimulates selectively myogenic cells in primary muscle culture. J Neuropathol Exp Neurol 54:121–128.

Cantini M, Massimino ML, Bruson A, Catani C, Dalla Libera L, Carraro U. 1994. Macrophages regulate proliferation and differentiation of satellite cells. Biochem Biophys Res Commun 202:1688–1696.

Cantini M, Massimino ML, Rapizzi E, Rossini K, Catani C, Dalla Libera L, Carraro U. 1995. Human satellite cell proliferation in vitro is regulated by autocrine secretion of IL-6 stimulated by a soluble factor(s) released by activated monocytes. Biochem Biophys Res Commun 216:49–53.

Cantini M, Giurisato E, Radu C, Tiozzo S, Pampinella F, Senigaglia D, Zaniolo G, Mazzoleni F, Vitiello L. 2002. Macrophage-secreted myogenic factors: A promising tool for greatly enhancing the proliferative capacity of myoblasts in vitro and in vivo. Neurol Sci 23:189–194.

Chazaud B, Sonnet C, Lafuste P, Bassez G, Rimaniol AC, Poron F, Authier FJ, Dreyfus PA, Gherardi RK. 2003. Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth. J Cell Biol 163:1133–1143.

Dudley GA, Czerkawski J, Meinrod A, Gillis G, Baldwin A, Scarpone M. 1997. Efficacy of naproxen sodium for exercise-induced dysfunction muscle injury and soreness. Clin J Sport Med 7:3–10.

Durant S, Duval D, Homo-Delarche F. 1989. Effect of exogenous prostaglandins and nonsteroidal anti-inflammatory agents on prostaglandin secretion and proliferation of mouse embryo fibroblasts in culture. Prostaglandins Leukot Essent Fatty Acids 38:1–8.

Fawthrop FW, Frazer A, Russell RG, Bunning RA. 1997. Effects of transforming growth factor beta on the production of prostaglandin E and caseinase activity of unstimulated and interleukin 1-stimulated human articular chondrocytes in culture. Br J Rheumatol 36:729–734.

Fiocchi C. 2001. TGF-beta/Smad signaling defects in inflammatory bowel disease: Mechanisms and possible novel therapies for chronic inflammation. J Clin Invest 108:523–526.

Goldstein RH, Polgar P. 1982. The effect and interaction of bradykinin and prostaglandins on protein and collagen production by lung fibroblasts. J Biol Chem 257:8630–8633.

Graf BA, Nazarenko DA, Borrello MA, Roberts LJ, Morrow JD, Palis J, Phipps RP. 1999. Biphenotypic B/macrophage cells express COX-1 and up-regulate COX-2 expression and prostaglandin E(2) production in response to pro-inflammatory signals. Eur J Immunol 29:3793–3803.

Haller D, Holt L, Kim SC, Schwabe RF, Sartor RB, Jobin C. 2003. Transforming growth factor-beta 1 inhibits non-pathogenic Gram negative bacteria-induced NF-kappa B recruitment to the interleukin-6 gene promoter in intestinal epithelial cells through modulation of histone acetylation. J Biol Chem 278:23851–23860.

Han C, Demetris AJ, Liu Y, Shelhamer JH, Wu T. 2004. Transforming growth factor-beta (TGF-beta) activates cytosolic phospholipase A2alpha (cPLA2alpha)-mediated prostaglandin E2 (PGE)2/EP1 and peroxisome proliferator-activated receptor-gamma (PPAR-gamma)/Smad signaling pathways in human liver cancer cells. A novel mechanism for subversion of TGF-beta-induced mitoinhibition. J Biol Chem 279:44344–44354.

Hasson SM, Daniels JC, Divine JG, Niebuhr BR, Richmond S, Stein PG, Williams JH. 1993. Effect of ibuprofen use on muscle soreness, damage, and performance: A preliminary investigation. Med Sci Sports Exerc 25:9–17.

Horsley V, Pavlath GK. 2003. Prostaglandin F2(alpha) stimulates growth of skeletal muscle cells via an NFATC2-dependent pathway. J Cell Biol 161:111–118.

Hui AY, Dannenberg AJ, Sung JJ, Subbaramaiah K, Du B, Olinga P, Friedman SL. 2004. Prostaglandin E2 inhibits transforming growth factor beta 1-mediated induction of collagen alpha 1(I) in hepatic stellate cells. J Hepatol 41:251–258.

Kahari VM, Larjava H, Uitto J. 1991. Differential regulation of extracellular matrix proteoglycan (PG) gene expression. Transforming growth factor-beta 1 up-regulates biglycan (PGI), and versican (large fibroblast PG) but down-regulates decorin (PGII) mRNA levels in human fibroblasts in culture. J Biol Chem 266:10608–10615.

Keerthisingam CB, Jenkins RG, Harrison NK, Hernandez-Rodriguez NA, Booth H, Laurent GJ, Hart SL, Foster ML, McAnulty RJ. 2001. Cyclooxygenase-2 deficiency results in a loss of the anti-proliferative response to transforming growth factor-beta in human fibrotic lung fibroblasts and promotes bleomycin-induced pulmonary fibrosis in mice. Am J Pathol 158:1411–1422.

Khalil N, Bereznay O, Sporn M, Greenberg AH. 1989. Macrophage production of transforming growth factor beta and fibroblast collagen synthesis in chronic pulmonary inflammation. J Exp Med 170:727–737.

Khalil N, Whitman C, Zuo L, Danielpour D, Greenberg A. 1993. Regulation of alveolar macrophage transforming growth factor-beta secretion by corticosteroids in bleomycin-induced pulmonary inflammation in the rat. J Clin Invest 92:1812–1818.

Kulkarni AB, Karlsson S. 1997. Inflammation and TGF beta 1: Lessons from the TGF beta 1 null mouse. Res Immunol 148:453–456.

Lee SK, Hong CH, Huh SK, Kim SS, Oh OJ, Min HY, Park KK, Chung WY, Hwang JK. 2002. Suppressive effect of natural sesquiterpenoids on inducible cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) activity in mouse macrophage cells. J Environ Pathol Toxicol Oncol 21:141–148.

Lescaudron L, Peltekian E, Fontaine-Perus J, Paulin D, Zampieri M, Garcia L, Parrish E. 1999. Blood borne macrophages are essential for the triggering of muscle regeneration following muscle transplant. Neuromuscul Disord 9:72–80.

Li Y, Foster W, Deasy BM, Chan Y, Prisk V, Tang Y, Cummins J, Huard J. 2004. Transforming growth factor-beta1 induces the differentiation of myogenic cells into fibrotic cells in injured skeletal muscle: A key event in muscle fibrogenesis. Am J Pathol 164:1007–1019.

McAnulty RJ, Hernandez-Rodriguez NA, Mutsaers SE, Coker RK, Laurent GJ. 1997. Indomethacin suppresses the anti-proliferative effects of transforming growth factor-beta isoforms on fibroblast cell cultures. Biochem J 321:639–643.

Merly F, Lescaudron L, Rouaud T, Crossin F, Gardahaut MF. 1999. Macrophages enhance muscle satellite cell proliferation and delay their differentiation. Muscle Nerve 22:724–732.

Mishra DK, Friden J, Schmitz MC, Lieber RL. 1995. Anti-inflammatory medication after muscle injury. A treatment resulting in short-term improvement but subsequent loss of muscle function. J Bone Joint Surg Am 77:1510–1519.

Monteleone G, Mann J, Monteleone I, Vavassori P, Bremner R, Fantini M, Del Vecchio Blanco G, Tersigni R, Alessandroni L, Mann D, Pallone F, MacDonald TT. 2004a. A failure of transforming growth factor-beta1 negative regulation maintains sustained NF-kappaB activation in gut inflammation. J Biol Chem 279:3925–3932.

Monteleone G, Pallone F, MacDonald TT. 2004b. Smad7 in TGF-beta-mediated negative regulation of gut inflammation. Trends Immunol 25:513–517.

Nakao A, Miike S, Hatano M, Okumura K, Tokuhisa T, Ra C, Iwamoto I. 2000. Blockade of transforming growth factor beta/Smad signaling in T cells by overexpression of Smad7 enhances antigen-induced airway inflammation and airway reactivity. J Exp Med 192:151–158.

Obremsky WT, Seaber AV, Ribbeck BM, Garrett WE, Jr. 1994. Biomechanical and histologic assessment of a controlled muscle strain injury treated with piroxicam. Am J Sports Med 22:558–561.

Pavlath GK, Horsley V. 2003. Cell fusion in skeletal muscle—Central role of NFATC2 in regulating muscle cell size. Cell Cycle 2:420–423.

Prisk V, Huard J. 2003. Muscle injuries and repair: The role of prostaglandins and inflammation. Histol Histopathol 18:1243–1256.

Qu Z, Balkir L, van Deutekom JC, Robbins PD, Pruchnic R, Huard J. 1998. Development of approaches to improve cell survival in myoblast transfer therapy. J Cell Biol 142:1257–1267.

Rando TA, Blau HM. 1994. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. J Cell Biol 125:1275–1287.

Robertson TA, Maley MA, Grounds MD, Papadimitriou JM. 1993. The role of macrophages in skeletal muscle regeneration with particular reference to chemotaxis. Exp Cell Res 207:321–331.

Saltzman LE, Moss J, Berg RA, Hom B, Crystal RG. 1982. Modulation of collagen production by fibroblasts. Effects of chronic exposure to agonists that increase intracellular cyclic AMP. Biochem J 204:25–30.

Seiler P, Aichele P, Odermatt B, Hengartner H, Zinkernagel RM, Schwendener RA. 1997. Crucial role of marginal zone macrophages and marginal zone metallophils in

the clearance of lymphocytic choriomeningitis virus infection. Eur J Immunol 27: 2626–2633.
Shen W, Li Y, Tang Y, Cummins J, Huard J. 2005. NS-398, a Cyclooxygenase-2-Specific Inhibitor, Delays Skeletal Muscle Healing by Decreasing Regeneration and Promoting Fibrosis. Am J Pathol 167:1105–1117.
Taipale J, Miyazono K, Heldin CH, Keski-Oja J. 1994. Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein. J Cell Biol 124:171–181.
Tidball JG. 2005. Inflammatory processes in muscle injury and repair. Am J Physiol Regul Integr Comp Physiol 288:R345–R353.
Tyner JW, Uchida O, Kajiwara N, Kim EY, Patel AC, O'Sullivan MP, Walter MJ, Schwendener RA, Cook DN, Danoff TM, Holtzman MJ. 2005. CCL5-CCR5 interaction provides antiapoptotic signals for macrophage survival during viral infection. Nat Med 11:1180–1187.
Wang W, Huang XR, Li AG, Liu F, Li JH, Truong LD, Wang XJ, Lan HY. 2005. Signaling Mechanism of TGF-{beta}1 in Prevention of Renal Inflammation: Role of Smad7. J Am Soc Nephrol 16:1371–1383.
Warshamana GS, Pociask DA, Fisher KJ, Liu JY, Sime PJ, Brody AR. 2002. Titration of non-replicating adenovirus as a vector for transducing active TGF-beta1 gene expression causing inflammation and fibrogenesis in the lungs of C57BL/6 mice. Int J Exp Pathol 83:183–201.
Wolff RA, Tomas JJ, Hullett DA, Stark VE, van Rooijen N, Hoch JR. 2004. Macrophage depletion reduces monocyte chemotactic protein-1 and transforming growth factor-beta1 in healing rat vein grafts. J Vasc Surg 39:878–888.
Yang X, Letterio JJ, Lechleider RJ, Chen L, Hayman R, Gu H, Roberts AB, Deng C. 1999. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. Embo J 18:1280–1291.

Blocking Myostatin by AAV2-Delivered Myostatin Propeptide Improves Muscle Cell Transplantation

* **Zhu J; *Ma J; ‡Qiao C, ‡Li J; §*Li Y, ‡Xiao, X; +*Huard J
*Stem Cell Research Center, Children's Hospital of Pittsburgh of UPMC and §Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA
jhuard@pitt.edu

INTRODUCTION

Myostatin (MSTN) is a potent negative regulator of muscle growth [1] and we have found that like TGF-β1, MSTN also contributes to the formation of fibrosis in injured skeletal muscle [2]. JacqueTremblay's group has successfully blocked MSTN signaling in *mdx* mice by generating 2 types of transgenic *mdx* mice. The 1st carries a dominant negative form of the MSTN receptor (dnActRIIB) and the 2nd over-expresses follistatin (FSTN), which is an inhibitor of MSTN. Normal myoblasts transplanted into these transgenic *mdx* mice outperformed myoblasts transplanted into nontransgenic *mdx* mice [3, 4]; nevertheless, dnActRIIB and FLST do not specifically inhibit MSTN but also interfere with other growth factors such as activins. In the current study we utilized an adeno-associated viral vector carrying the MSTN propeptide gene (AAV-MPRO) to specifically inhibit the action of MSTN. MPRO directly binds the MSTN molecule and has been shown in previous studies. We have proved that blocking MSTN with MPRO both increase muscle regeneration and reduce the formation of fibrosis at the site of injury 4 weeks following injury (unpublished data). In the current study we injected AAV-MPRO/GPF into the skeletal muscles of *mdx*/SCID mice 4 weeks prior to muscle progenitor cell transplantation to investigate whether blocking MSTN signaling in host muscle by MPRO can elevate the regeneration capacity of donor muscle cells in dystrophic muscle. The success of cell therapy for treating muscle injuries and diseases has been limited by the poor survival and function of the donor cells after transplantation; therefore, improving the microenvironment in the host dystrophic muscle prior to cell transplantation is an alternative approach to enhance the efficiency of cell transplantation.

METHODS

Animal model: All experiments in this study were approved by the Children's Hospital of Pittsburgh IACUC. AAV2-MPRO ($1x10^{11}$ v.g.) in 50 µl of PBS was injected into the gastrocnemius muscles (GMs) of 3 *mdx*/SCID mice (4 weeks of age); the same amount of AAV-GFP was injected into the GMs of mdx/SCID littermates as controls. Four weeks after injection of AAV, 300,000 muscle progenitor cells (MPCs) were injected into each of the GMs of AAV treated *mdx*/SCID mice (n = 6), and the mice were sacrificed 2 weeks following the cell transplantation. Masson's Trichrome staining was performed to identify fibrous scar tissue in the mice. Northern Eclipse software (Empix Imaging, Inc.) was used to measure the diameters of regenerating myofibers within the injection site and also measure the areas of fibrous scar tissue. Dystrophin staining was performed and the dystrophin-positive myofibers were then counted to assess the efficiency of cell transplantation in the skeletal muscle of the *mdx*/SCID mice. Student's *t* test was used to determine significance ($P<0.05$).

Immunohistochemistry: Muscles were snap frozen in liquid nitrogen, cyrosectioned and the tissue sections were then fixed in 5 % formalin for 5 minutes, followed by 3 washes with PBS. The sections were blocked with 10% donkey serum for 1 hour, and incubated overnight at 4°C in either a goat MPRO antibody (2.5 ug/ml, RnD system) or a rabbit dystrophin primary antibody (1:500, Abcam) that was diluted in 5% donkey serum. The following day, the sections were washed with PBS and incubated with the corresponding secondary antibodies, rabbit anti-Goat or donkey anti-rabbit IgG conjugated with Alexa594.

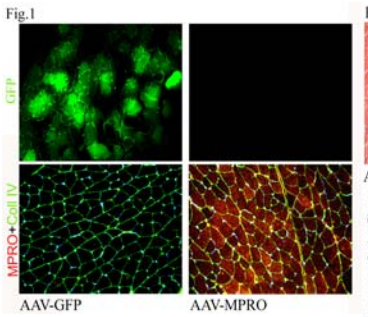
RESULTS

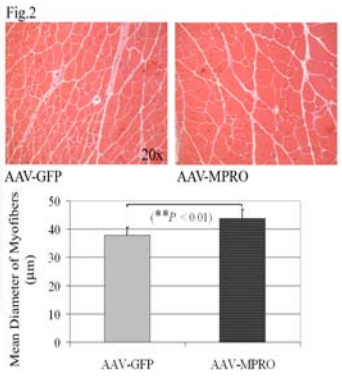
Gene expression in muscle: Expressions of GFP (green) and MPRO (red) in AAV-GFP/MPRO transduced muscles indicates that genes delivered by AAV were stably expressed in skeletal muscle (Fig. 1).

Accelerated muscle regeneration in MPRO over-expressing mdx/SCID mice: Six weeks after AAV2-MPRO transduction, a significant increase in muscle weight was observed compared to the GFP transduced control (data not shown). Moreover, the mean diameter of the muscle fibers in the AAV-MPRO transduced muscles was significantly larger than that of AAV-GFP transduced muscles (Fig. 2).

Reduced fibrosis: Masson's trichrome stain showed a reduction of fibrous scar tissue (blue) in the AAV2-MPRO transduced GMs as compared to the AAV-GFP control muscles (Fig. 3).

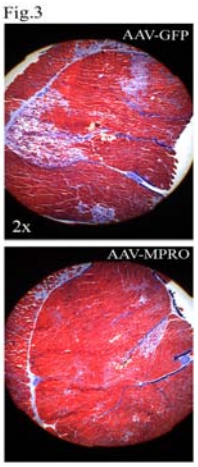
Improved cell transplantation in the GMs of AAV-MPRO transduced mdx/SCID mice: Fig.4 shows the representative images of dystrophin-positive muscle fibers in the muscles of *mdx*/SCID mice 2 weeks after cell transplantation. Normal MPCs injected into AAV-MPRO transduced dystrophic muscle surpassed the regeneration capacity of cells injected into AAV-GFP transduced control muscle. This was determined by counting the total number of dystrophin positive myofibers (Fig. 4)

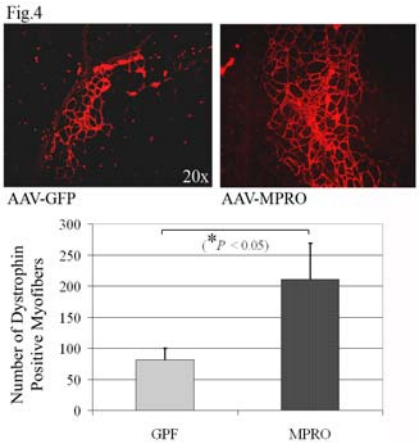




DISCUSSION

Our results revealed stable expression of AAV-mediated GFP and MPRO in transduced skeletal muscle. MPRO ameliorated the dystrophic pathology of *mdx* mice by promoting muscle regeneration and reducing collagen deposition. Inactivation of MSTN in dystrophic host muscle by MPRO significantly improved the success of MPC transplantation as compared to the AAV-GFP transduced control, evidenced by significantly more dystrophin-positive myofibers in the former. Two mechanisms may account for this improved cell transplantation. First, MPRO counteracts the inhibitory effect that MSTN has on donor cell regeneration. Deregulating the suppression of MSTN on muscle cells by MPRO transduction may augment donor cell proliferation, differentiation, and hence increase dystrophin-positive myofiber formation. Second, the improved cell transplantation in *mdx* mice by MPRO may be partially accredited to the fact that MPRO inhibits MSTN, thereby reducing fibrosis formation in dystrophic muscle. The ameliorated milieu in host dystrophic muscle may favor donor cell survival and engraftment. Taken together, the combination of gene therapy and cell therapy has been shown to be a potential effective and novel approach for treating injured and diseased muscle.





ACKNOWLEDGMENTS

This work was supported by funding from the Henry J. Mankin Endowed Chair for Orthopaedic Research at the University of Pittsburgh, the William F. and Jean W. Donaldson Chair at Children's Hospital of Pittsburgh, the Hirtzel Foundation, and the National Institutes of Health (R01 AR47973 awarded to J.H.).

REFERENCES

1. McPherron AC et al. Nature 1997;387:83–90. 2. Zhu J et al. J Biol Chem 2007 ; 282(35):25852-63. 3. Benabdallah et al. Transplantation 2005; 79(12):1696-702. 4. Benabdallah et al. Cell Transplant 2008;17(3):337-50.

AFFILIATED INSTITUTIONS FOR CO-AUTHORS

**Department of Bioengineering, University of Pittsburgh, ‡University of North Carolina School of Pharmacy

Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing in a Dose Dependent Manner

*Uehara, K; *Nozaki, M; *Zhu J; *Quintero, AJ; *Ota, S; **Fu, FH; +*Huard, J
* Stem Cell Research Center, Children's Hospital of Pittsburgh of UPMC and **Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA
jhuard@pitt.edu

INTRODUCTION:

Muscle injuries are very common musculoskeletal problems encountered in sports medicine. Although these injuries are capable of healing, complete functional recovery is hindered by the formation of dense scar tissue triggered by TGF-β1 [1]. We have previously reported that several agents such as decorin and suramin can inhibit fibrosis and improve regeneration in injured skeletal muscle. The safety of these agents, however, remains unknowns to use those agents for treating muscle injury. By contrast, Losartan (LOS), one of the Angiotensin II Receptor Blockers (ARBs)—is a FDA approved antihypertensive medication and has been shown to also be antifibrotic in a variety of tissues, including skeletal muscle [2]. This ARB has a well-tolerated side-effect profile, can also block TGF-β1 to attenuate the development of pathological fibrosis. In this study, we investigated optimum dose of LOS in treating injured muscle to help the translation of this research from bench to bedside.

METHODS:

Cell proliferation assay: C2C12 myoblasts and fibroblasts obtained from murine skeletal muscle by using the previously published preplating technique [3], were cultured in 96 well plates (n=5) with DMEM containing 10% FBS,1% penicillin/streptomycin (P/S), with addition of different concentration of either Angiotensin II (ANG) (10^{-12} to 10^{-4} M) or LOS (10^{-12} to 10^{-4} M). CellTiter Cell Proliferation Assay kit (Promega, Madison, WI) was used to measure cell proliferation at 24, 48, and 74 hour after incubation.

C2C12 cell differentiation assay: C2C12 myoblasts were cultured in 24 well plates (n=4) with differentiation medium (DMEM supplemented 2% horse serum and 1% P/S) containing different concentration of ANG (10^{-12} to 10^{-4} M) or LOS (10^{-12} to 10^{-4} M). Three days after incubation immunocytochemistry of myosin heavy chain was performed and the fusion index (a ratio of the number of nuclei in myotubes/total nuclei) was calculated to assess the effects of ANG and LOS on differentiation capacity of C2C12 myoblast.

Animal model: The muscle contusion model was developed in tibialis anterior (TA) muscle of C57BL/6 wild-type mice. Different concentrations of LOS (12.5, 125, 1250, or 4000mg) in 1Liter of tap water were administered beginning immediately after injury until endpoint while the control group drink tap water (six mice in each group). These doses were calculated based on the average fluid intake of mice as 3, 30, 300, and 1000mg/kg/day respectively. Animals were sacrificed and TA muscles were harvested 4 weeks after injury. Muscle samples were then cryosectioned and histological stained (hematoxlin and eosin stain (H&E) and Masson's Trichrome stain). The numbers of centronucleated regenerating myofibers were counted to evaluate the regeneration.Northern Eclipse software (Empix Image, Inc.) was used to quantitate the total cross-sectional area of fibrosis. Statistical analysis was performed with ANOVA and Scheffe's F test as post hoc test. Statistical significance was defined as $p < 0.05$.

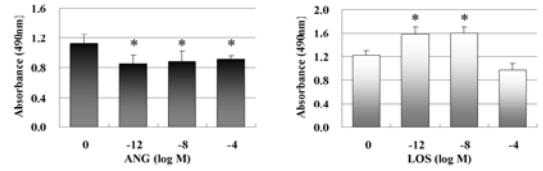
RESULTS SECTION:

LOS enhanceed C2C12 myoblast proliferation: Exposure to 10^{-12} to 10^{-4} M of ANG attenuated C2C12 proliferation after 72hr incubation. Whereas, 10^{-12} to 10^{-8} M of LOS stimulated C2C12 proliferation at the same time point. However, a high concentration of LOS (10^{-4} M) failed to increase cell growth (Fig. 1). Surprisingly, neither ANG nor LOS had any effect on fibroblast proliferation (not shown).

LOS stimulated myoblast differentiation: C2C12 differentiation was inhibited by 10^{-12}M of ANG, however, the effect attenuated at higher concentrations (Fig.2). Likewise, 10^{-12} M of LOS enhanced C2C12 differentiation and the stimulation was dropping while concentrations of LOS increased. It is noteworthy that 10^{-4} M of LOS significantly suppressed differentiation (Fig. 2).

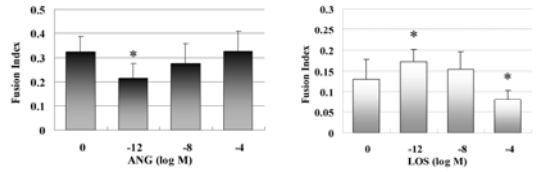
LOS enhanced muscle regeneration and decreased fibrosis: We could observe significant increases in number of centronucleated myofibers in the LOS-treated animals when compared with control animals (Fig. 3). The group treated with 300 mg/kg/day of LOS showed the most effective regeneration among groups. The best regeneration correlated with the group that displayed a significant reduction in fibrosis. However, these beneficial effects decreased when the dose of LOS was increased to 1000 mg/kg/day (Fig. 4).

Figure. 1



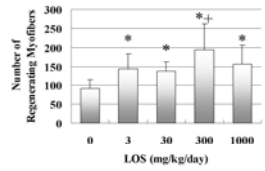
*: $p < 0.05$ vs control

Figure. 2



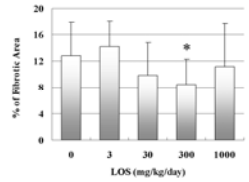
*: $p < 0.05$, vs control

Figure. 3



*: $p < 0.05$, vs control
+: $p < 0.05$, vs other trreated group

Figure. 4



*: $p < 0.05$, vs control

DISCUSSION:

The biphasic effect of LOS on C2C12 myoblasts *in vitro*, stimulating at low dose while decreasing at high dose, suggested that there is an optimal dose of LOS. Consequently we found that LOS improves skeletal muscle regeneration at 4 weeks after contusion injury, except that these effects were reduce/eliminated in 1000 mg/kg/day group. The best effective dose was 300 mg/kg/day. Overall, these effects of LOS were more pronounced for regeneration than for fibrosis. These *in vivo* results are consistent with our *in vitro* results that LOS was able to exert effects on C2C12 whereas fibroblasts were not affected by either ANG or LOS. Regeneration and fibrosis are two competitive processes after muscle injury; therefore, decreasing fibrosis observed in LOS-treated group is can be the result of the increase regeneration. In other words, LOS might indirectly reduced fibrosis by directly stimulating regeneration. Although there were not statistical differences in fibrosis in lower dose LOS groups (3 and 30 mg/kg/day) as compared to control, these effects of LOS on both regeneration and fibrosis showed similar dose dependent trend. However, *in vivo* results above were obtained only from single time point after injury. Since there are time lags between peaks of myofiber regeneration and fibrosis after injury, further investigations are required to examine the effect of LOS on regeneration and fibrosis at their individual peak time, which will facilitate clinical application of ARBs in improving skeletal muscle healing.

ACKNOWLEDGEMENTS:

The authors are grateful for technical assistance from Jessica Tebbets, and Aiping Lu. Funding support was provided by a grant from the Department of Defense (W81XWH-06-1-0406).

REFERENCES:

1. Li Y, *et al*., *Am J Pathol*. 2004;164: 1007-19.
2. Bedair HS, *et al*., *Am J Sport Med*. 2008; 36: 1548-54.
3. Qu Z, *et al*., *J Cell Biol*. 1998; 142: 1257-67.

Blocking Myostatin Improves Muscle Healing Via Enhancement of Angiogenesis

* **Zhu J; *Ma J, ‡Qiao C; ‡Li J; *§Li Y; ‡Xiao, X; +*§Huard. J
+*Stem Cell Research Center, Children's Hospital of Pittsburgh of UPMC and §Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA
jhuard@pitt.edu

INTRODUCTION

Myostatin (MSTN), a member of the TGF-β superfamily, is a powerful inhibitor of skeletal muscle growth [1], and has also been implicated in the formation of fibrosis in injured skeletal muscle [2]. An increase of muscle regeneration and a decrease of fibrosis is observed in the injured muscle of MSTN knockout (MSTN-/-) mice when compared to that of injured wild-type (WT) mice [2]. We have also observed similar results when we block MSTN utilizing adeno-associated viral vector to deliver MSTN propeptide (AAV-MPRO) (unpublished data), a natural inhibitor of MSTN [3]. In this study, we investigated whether AAV-MPRO could stably express in vivo and benefit the healing of injured skeletal muscle up to one year after muscle injury. We further examined mechanisms by which the inhibition of MSTN improved skeletal muscle healing after laceration injury. A positive correlation has been reported between angiogenesis and skeletal muscle healing [4]; therefore, we hypothesized that injured MSTN-/- muscles and injured muscles transduced with AAV-MPRO would show more angiogenesis than their respective controls.

METHODS

Animal experiments: We injected AAV-MPRO ($1x10^{11}$ v.g.) in 50 µl of PBS into gastrocnemius muscles (GMs) of C57BL/6 WT mice (6-8 weeks old). AAV-GFP was injected into GMs of WT littermates as a control. One month after AAV vector transduction, the GMs of the mice were lacerated. The GMs of 10 mice were harvested at 4 weeks post-injury (n = 5). The remaining mice were sacrifice at 1 year post-injury (n = 4). HE staining was performed to monitor muscle regeneration in the injured muscles. All samples were stored in -80°C.

Immunohistochemistry: The muscles were cryosectioned and fixed in formalin. The sections were then incubated with 10% HS for 1 hour after which a rat CD31 (endothelial marker) primary antibody (1:150) was applied along with a mouse anti-fast myosin heavy chain (MyHC) antibody or sheep anti-MPRO antibody, which were incubated for 1 hour at RT. The sections were then washed three times with PBS and incubated with the secondary antibodies rabbit anti-rat conjugated with 555 (red) and anti-mouse conjugated with 488 (green) or anti-sheep-555 (red) for 1h. Nuclei were counter stained with DAPI (blue). We used image J software to measure the number of CD31-positive capillaries to evaluate angiogenesis within the injury site. Student's *t* test was used to determine significance ($P<0.05$).

RESULTS

Expression of GFP and MPRO in skeletal muscle: One year after AAV transduction, we still detected strong expressions of GFP and MPRO in myofibers of AAV-GFP/MPRO transduced muscles (Fig. 1). MPRO-positive myofibers (arrowheads) indicates constitutive expression of MPRO in skeletal muscle transduced with AAV-MPRO.

Improved skeletal muscle healing by MPRO: We observed significantly larger regenerating myofibers in AAV-MPRO transduced injured muscle than in controls, 1 year post-injury (Fig. 2A, C). Correspondingly, distribution of diameter of regenerating myofibers revealed that AAV-MPRO transduced injured muscle contained a higher percentage of larger diameter regenerating myofibers. For instance, 30% of the diameters of regenerating myofibers in control muscle were larger than 50 µm when compared to 61 % of that in AAV-MPRO transduced muscle (Fig. 1B). Blocking MSTN with MPRO led to a significant increase in the weight of the GMs (Fig. 2D).

Increased angiogenesis in AAV-MPRO-transduced mice after GM laceration: Fig.3 shows representative images from injured AAV-MPRO transduced muscles and controls. Significantly more CD31-positive capillaries were observed in AAV-MPRO transduced injured muscle than in controls at 4 weeks post-injury (Fig.3).

DISCUSSION

Our unpublished data show that angiogenesis occurs earlier in the MSTN-/- muscle than in normal muscles after laceration injury. At 4 weeks after injury, MSTN-/- muscle contained significantly more CD31-positive capillaries than the controls. The increased angiogenesis appears to partially account for improved muscle healing in injured MSTN-/- mice. Nevertheless, it is noteworthy that the irreversibility of genetic transfer may result in compensatory upregulation, developmental defects, etc. We therefore used AAV to deliver MPRO cDNA into the GMs of WT mice in order to restrict MSTN propeptide over-expression in the certain skeletal muscles of adult mice. With this model, we injected AAV-MPRO particles into skeletal muscle prior to creating a laceration injury in the mice. Our results suggest that AAV-MPRO could be stably expressed in vivo and could improve the healing of the injured muscle, long term. We found that MPRO gene transfer improved skeletal muscle healing with increased muscle regeneration. Muscle transduced with MPRO also showed a greater amount of angiogenesis than untransduced controls which coincided with the increased regeneration and decreased fibrosis 4 weeks after injury (data not shown). Taken together, this data suggests a negative correlation between MSTN and angiogenesis in injured skeletal muscle; however, the clear relationship between MSTN and angiogenesis warrants further investigation.

Fig.1

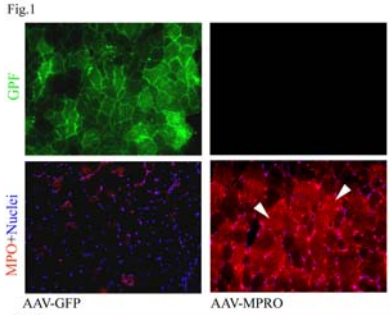


Fig.2

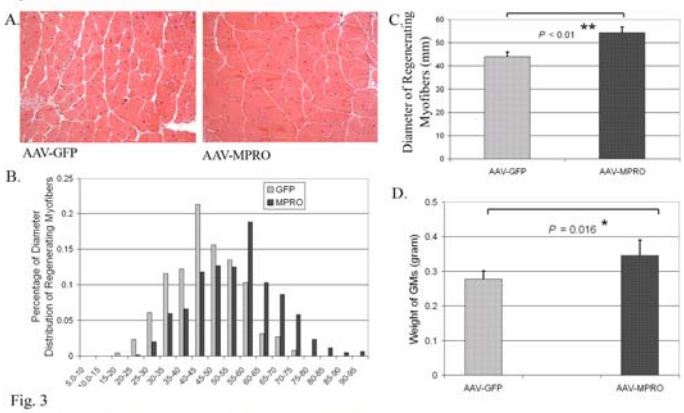
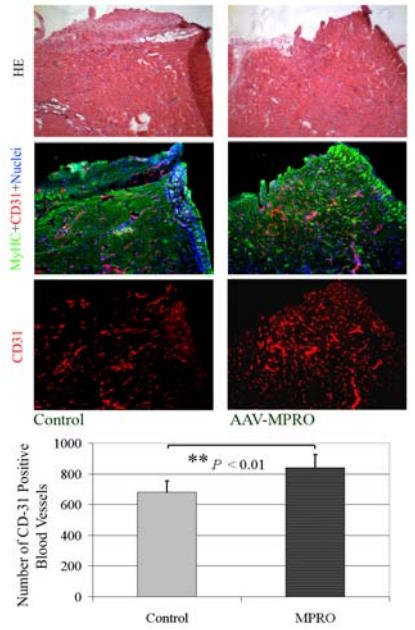


Fig. 3



ACKNOWLEDGMENTS

This work was supported by funding from the Henry J. Mankin Endowed Chair for Orthopaedic Research at the University of Pittsburgh, the William F. and Jean W. Donaldson Chair at Children's Hospital of Pittsburgh, the Hirtzel Foundation, and the National Institutes of Health (R01 AR47973 awarded to J.H.).

REFERENCES

1. McPherron AC et al. Nature 1997;387:83–90. 2. Zhu J et al. J Biol Chem 2007 ; 282(35):25852-63. 3. Lee SJ et al. Proc Natl Acad Sci USA 2001;98:9306–11. 4.Nguyen et al. Neuromuscul Disord. 15 (2):154-63, 2005.

AFFILIATED INSTITUTIONS FOR CO-AUTHORS

**Department of Bioengineering, University of Pittsburgh, ‡University of North Carolina School of Pharmacy

Angiotensin Receptor Blocker Improves Skeletal Muscle Function Recovery in a Dose Dependent Manner

* †Uehara, K; *Kobayashi, T; *Ota, S; **Bin, S; **Tobita, K; ***Ambrosio, F; ‡Fu, FH; *Huard, J
* Stem Cell Research Center, Children's Hospital of Pittsburgh, **Department of Pediatrics, Children's Hospital of Pittsburgh, ***Department of Physical Medicine and Rehabilitation, University of Pittsburgh and ‡Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA, †Department of Orthopaedic Surgery, St. Marianna University School of Medicine, Japan
jhuard@pitt.edu

INTRODUCTION:
Muscle injuries are very common musculoskeletal problem encountered in sports medicine. Although these injuries are capable of healing, complete functional recovery is hindered by the formation of dense scar tissue triggered by TGF-β1 [1]. We have previously reported that Losartan (LOS), one of the Angiotensin II Receptor Blockers (ARBs) FDA approved for antihypertensive treatment, has been shown to improve muscle healing through antifibrotic action [2]. We also demonstrated that specific doses of LOS (30 mg/kg/day and higher) improved muscle regeneration and attenuated the development of pathological fibrosis when were administrated immediately after injury [3]. In this study, we investigated whether LOS can improve muscle strength recovery after contusion injury, and also attempted to understand the mechanism of LOS action by analyzing gene expression of myostatin and follistatin, which are considered important regulators of skeletal muscle growth.

METHODS:

Animal model: The muscle contusion was developed in tibialis anterior (TA) muscle of C57BL/6 wild-type mice. Different concentrations of LOS (12.5, 125, 1250, or 4000mg dissolved in 1 liter of tap water) were administered to animals immediately after injury (n=6 in each group). These doses were calculated based on the average fluid intake of mice as 3, 10, 30, 300 mg/kg/day respectively. The control-injured mice received tap water. After 4 weeks animals were sacrificed to evaluate the healing.

Physiological testing: Under general anesthesia, the TA distal tendon was exposed and tied securely to a lever arm of transducer. Peroneal nerve was electrically stimulated and the specific peak twitch and tetanic force developed by TA muscle was monitored. All data were digitally recorded and stored until evaluation.

Quantitative Real-time PCR: Total RNA was extracted from muscle tissues using Nucleospin RNA kit (Clontech). cDNA was synthesized with SuperScriptTM II reverse transcriptase (Invitrogen), according to manufacturer's instructions. cDNAs and primers were added to SYBR Green PCR master mix (Applied Biosystem) according to manufacturer's instructions. The quantitative analysis for follistatin and myostatin gene expression was performed. All data were normalized to cyclophilin which was used as the internal control.

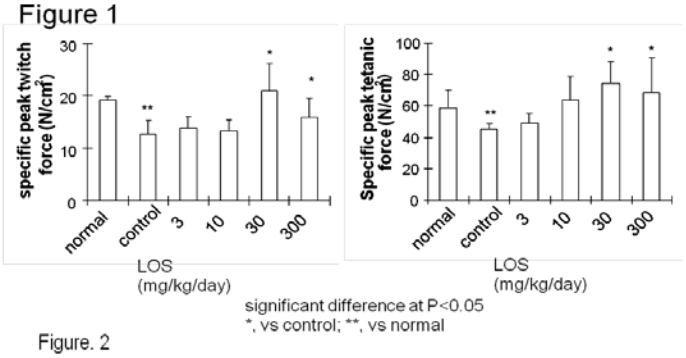
Statistical analysis: The results were expressed as the mean ± SD. To determine minimum effective dose of LOS in physiological testing, we analyzed the results using Williams' multiple comparison. The differences of mean specific twitch and tetanic force between control muscle (injured/non-treated) and normal muscle (non-injured) were analyzed with student t-test. Differences of gene expression between samples were assessed by the ANOVA and Tukey's test as post hoc test. Statistical difference was defined as $p < 0.05$.

RESULTS SECTION:

Improvement of Muscle Strength: LOS improved TA muscle force recovery after contusion injury. Specific peak twitch force and tetanic force was elevated in mice receiving high dose of LOS (30 and 300 mg/kg/day) in comparison to the animals receiving low dose of LOS (3 and 10 mg/kg/day) (Figure 1).

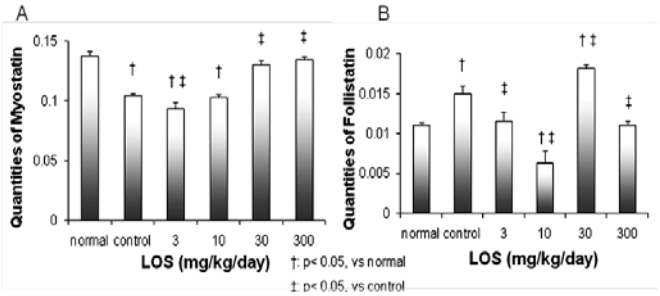
Myostatin gene expression: Expression of myostatin in the injured TA muscle in the control-injury and low dose of LOS treatment groups was lower than in the normal TA muscle, while there was no difference between normal and high dose of LOS treatment groups. Expression of follistatin in the control group was higher than in normal muscle and low dose of LOS treatment groups. The highest expression of follistatin was observed in the 30 mg/kg/day of LOS treatment group and it was significantly higher compared to the control group (Figure 2).

Figure 1



significant difference at P<0.05
*, vs control; **, vs normal

Figure. 2



DISCUSSION:
Functional recovery is the most important factor in the skeletal muscle healing after injury. Here we demonstrate that LOS administration immediately after injury improves recovery of skeletal muscle strength. These results also support our previous histological findings [3]. We believe that the mechanism of muscle regeneration after injury might be related to the expression of follistatin, positive regulator of skeletal muscle growth. We noticed over expression of follistatin in the 30 mg/kg/day treatment group compared to the normal and control-injury groups. These findings correlate with the results of physiological testing. It is unclear why the 30 mg/kg/day LOS display on increase expression of myostatin, negative regulator of skeletal muscle growth. Our results suggest that continuous administration of the high dose of LOS, in particularly 30 mg/kg/day, immediately after skeletal muscle injury could accelerate skeletal muscle functional recovery. We aimed to evaluate only single time point of LOS administration immediately after injury. Further studies are required to determine biological effect of LOS and facilitate clinical application of ARBs for improvement of skeletal muscle healing.

ACKNOWLEDGEMENTS:
The authors are grateful for technical assistance from Jessica Tebbets, Joseph Feduska, Michelle Witt, Richardo Ferrari, Avidas Usas and Burhan Gharaibeh. Funding support was provided by a grant from the Department of Defense (W81XWH-06-1-0406).

REFERENCES:

1. Li Y, *et al*., *Am J Pathol*. 2004;164: 1007-19.
2. Bedair HS, *et al*., *Am J Sport Med*. 2008; 36: 1548-54.
3. Uehara K, *et al*., 55th Annual Meeting of the Orthopaedic Research Society, 2009.

Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing

+[1]Kobayashi, T; [1]Uehara, K; [1]Ota, S; [2]Bin, S; [2]Tobita, K; [3]Ambrosio, F; [4]Fu, FH; [1]Huard, J
+[1] Stem Cell Research Center, Children's Hospital of Pittsburgh, [2]Department of Pediatrics, Children's Hospital of Pittsburgh, [3]Department of Physical Medicine and Rehabilitation, University of Pittsburgh and [4]Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA
jhuard@pitt.edu

INTRODUCTION:
Muscle injuries are very common musculoskeletal problem encountered in sports medicine. Although these injuries are capable of healing, complete functional recovery is hindered by the formation of dense scar tissue triggered by TGF-β1 [1]. Losartan (LOS) is a FDA approved antihypertensive medication and has a well-tolerated side-effect profile. Our previous study revealed that 30 mg/kg/day of LOS treatment was effective in promoting muscle healing and inducing antifibrotic effect in a murine model of skeletal muscle after injury [2,3]. However, the effective dose (30 mg/kg/day) which was administrated immediately after muscle injury is higher compared to the dose used in human (10 mg/kg/day). In this study we investigated the effect on muscle healing in a murine animal model using human dose of LOS (10 mg/kg/day) administered at different time after injury.

METHODS:

Animal model: The muscle contusion model was developed in tibialis anterior (TA) muscle of C57BL/6J wild-type mice. The administration of 125 mg of LOS dissolved in 1 liter of tap water was started on day 0, 3, 7 and 14 after injury and continued for 4 weeks. Tap water without LOS was given to the animals in control-injury group (6 mice in each group). The LOS dose taken was calculated based on the average fluid intake of mice [3]. Four weeks after injury, we performed physiological testing and harvested the TA muscle for histology.

Physiological testing: Under general anesthesia, the TA distal tendon was exposed and tied securely to a lever arm of transducer. Peroneal nerve was electrically stimulated and the specific peak twitch and tetanic force developed by TA muscle was monitored. All data were digitally recorded and stored until evaluation.

Histology: TA muscles harvested 4 weeks after injury were cryosectioned and stained for hematoxlin and eosin (H&E) and Masson's Trichrome stain. The number of centronucleated regenerating myofibers was counted to evaluate muscle regeneration. Northern Eclipse software (Empix Image, Inc.) was used to analyse the total cross-sectional area of muscle fibrosis.

Quantitative Real-time PCR: Total RNA was extracted from muscle tissues using Nucleospin RNA kit (Clontech). cDNA was synthesized with SuperScriptTM II reverse transcriptase (Invitrogen), according to manufacturer's instructions. cDNAs and primers were added to SYBR Green PCR master mix (Applied Biosystem) according to manufacturer's instructions. The quantitative analysis for Follistatin (FSTN) and Myostatin (MSTN) gene expression was performed. All data were normalized to cyclophilin which was used as the internal control.

Statistical analysis: Differences between samples were assessed by the ANOVA and Scheffe's F test as post hoc test. Statistical significance was defined as $p < 0.05$.

RESULTS:

LOS enhanced muscle regeneration and reduced fibrosis: We observed significant increases in the number of centronucleated myofibers in the day 3 treatment group when compared with other treatment groups (Fig. 1). The highest effect on muscle regeneration coincided with significant decrease of fibrosis in the day 3 treatment group (Fig. 2).

LOS enhanced muscle force: LOS improved muscle strength recovery after contusion injury. Specific peak twitch force and peak tetanic force was significantly greater in mice treated with LOS beginning at day 3 after injury (Fig. 3).

LOS enhanced expression of Folistatin and Myostatin: Expression of FSTN detected by RT-PCR in the day 3 and day 14 LOS treatment groups was greater than in the normal non-injured or LOS treated at day 0 and day 7 groups (Fig. 4A). Expression of MSTN in the day 3, day 7 and day 14 LOS treatment groups was lower than in the normal, control and day 0 LOS treatment groups (Fig. 4B). The highest expression of FSTN coincided with the lowest expression of MSTN in the day 3 LOS treatment group.

Figure 1

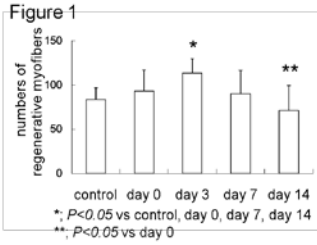


Figure 2

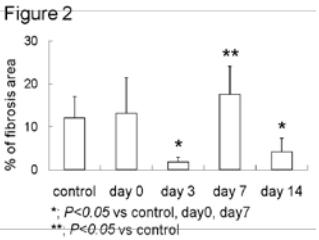


Figure 3

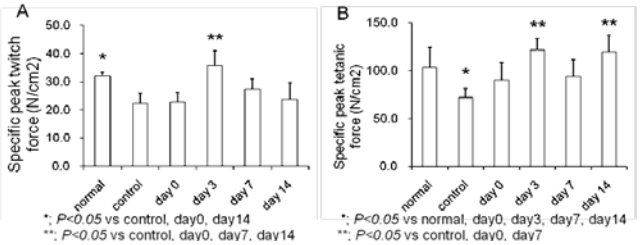
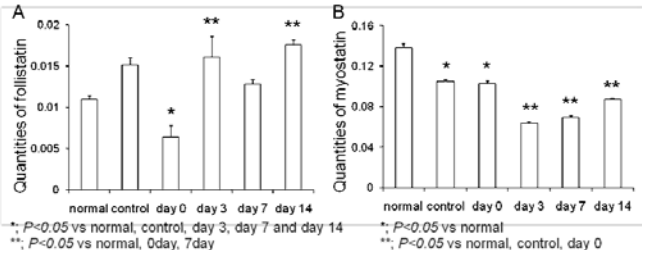


Figure 4



DISCUSSION:
Our study revealed that the most effective timing for administration of human dose of LOS (10 mg/kg/day) was 3 days after muscle injury. We observed increased number of centronucleated myofibers and decreased area of fibrosis when LOS was administered at day 3 after injury. The functional recovery after skeletal muscle injury is the most important factor for clinical translation of this therapy. We demonstrate that enhancement of muscle strength in day 3 LOS treatment group correlates with the improvement of muscle regeneration and the reduction of fibrosis. We have previously reported that muscle regeneration and fibrosis formation are two concomitant processes after muscle injury, and the effect of LOS was more prominent on muscle regeneration than on fibrosis [3]. Our study supports this finding. In addition, it may suggest that administration of LOS effectively leads to enhanced muscle regeneration after muscle injury via down regulation of endogenous MSTN, negative regulator of skeletal muscle growth. A decrease in FSTN expression was also observed in the day 3 LOS treatment group which may be related to the decrease in MSTN expression. In summary, we indicate that 10 mg/kg/day (human safety dose) of LOS treatment initiated at 3 days after contusion injury can enhance structural and functional healing in mouse skeletal muscle.

ACKNOWLEDGEMENTS:
The authors are grateful for technical assistance from Jessica Tebbets, Joseph Feduska, Michelle Witt, Richardo Ferrari, Aiping Lu, Avidas Usas and Burhan Gharaibeh. Funding support was provided by a grant from the Department of Defense (W81XWH-06-1-0406).

REFERENCES:

1. Li Y, *et al*., *Am J Pathol*. 2004; 164: 1007-19.
2. Bedair HS, *et al*., *Am J Sport Med*. 2008; 36: 1548-54.
3. Uehara K, *et al*., 55th Annual Meeting of the Orthopaedic Research Society, 2009.

Appendix 15

Improving Recovery Following Recurrent Hamstring Injury Using an Angiotensin II Receptor Blocker: Two Case Studies

Journal:	*Orthopaedic Research Society*
Abstract ID:	Draft
Presentation Type:	Either Poster or Podium
Categories for Abstract Review:	Muscle/Nerve - Muscle, Trauma - Clinical trauma research, Gait and Kinematics - Gait, Kinematics, Kinesiology
Keywords for Transactions on DVD-ROM:	Clinical Outcomes Research, Muscle, Gait, Kinematics, Kinesiology

Improving Recovery Following Recurrent Hamstring Injury Using an Angiotensin II Receptor Blocker: Two Case Studies

†*Yuri Chun MD, †* Sheila J M Ingham MD, *James Irrgang PhD, *Tanya Hagen MD, *Freddie Fu MD,
†*Burhan Gharaibeh, *Vonda Wright MD, †*Johnny Huard, PhD
†Stem Cell Research Center, Children's Hospital of Pittsburgh, Pittsburgh, PA
*Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA
jhuard@pitt.edu

INTRODUCTION

Hamstring muscle injuries are common in young competitive athletes. A recent study on National Football League training camp injuries reports hamstring injuries were the most common among muscle strain as well as the most severe. Recurrence is common, secondary to the pressure to return to play prior to complete healing of the injury, with a higher re-injury rate than any other type of injuries. Although muscles can undergo regeneration after injury, the healing process is slow and often culminates in incomplete functional recovery and formation of fibrosis.

A growing understanding of the cellular and molecular events that commonly occur during fibrosis in various tissues, including skeletal muscle, has provided a strong foundation for the development of effective therapies to prevent fibrosis and improve tissue healing. Because TGF-β1 plays such a crucial role in tissue fibrosis, particularly in skeletal muscle, it warrants attention as a key target for anti-fibrotic applications. Of the agents to block TGF-β1, Losartan potassium, a non-peptide molecule that works as an angiotensin II receptor blocker, is particularly attractive for clinical application as it is FDA approved and has minimal side effects. In a murine model, we have found that angiotensin receptor blocker-treated mice exhibited a histological, dose-dependent improvement in muscle regeneration and a significant reduction in fibrous tissue formation within the area of injury.

Given that Losartan has already been used clinically with an extremely safe side effect profile, we have conducted two case studies in young college athletes that sustained recurrent hamstring injuries and whose recoveries were safely improved with losartan. This is an off-label use of losartan (i.e.: the FDA has not approved labeling the device for the described purpose). Here we report the results obtained.

METHODS

Both subjects were submitted to the same protocol that is herein described: after obtaining the subjects' informed consent for treatment, both subjects were started on a 30-day treatment course of losartan at the manufacturer's recommended oral dose of 50 mg per day. Both subjects were healthy and had none of the contra-indications for the use of losartan. In addition to the medication, they underwent a routine rehabilitation program that gradually progressed to eccentric strengthening. The subjects reported no side effects while they were taking the study medication and remained normotensive throughout.

They were initially evaluated by clinical examination and were subsequently evaluated every 7 days with serial measurements (with a hand-held dynamometer - Lafayette Instrument Inc) of hamstring flexibility and strength as well as their blood pressure. Prior to the start of the medication the subjects were submitted to a magnetic resonance imaging (1.5T; GE-Sigma, Waukesha, WI, USA). After a period of 11 weeks the subjects underwent an isokinetic test (Biodex II) to better evaluate muscle strength compared to the non-injured side.

RESULTS

- Subjects

Subject #1: male, 21 years old, college athlete (football punter). He presented 10 days after an acute onset of "searing" pain in his left posterior thigh when he was kicking with his left leg. He referred a similar injury 5 weeks prior to the present injury.

Subject #2: male, 22 years old, college athlete (Ultimate Frisbee). He presented 4 days after an acute onset of pain in his left posterior thigh while he was sprinting. He referred two previous hamstring injuries (2 and 7 months prior to the present injury).

- MRI results (at time of injury)

Subject #1: Acute Grade 2 hamstring strain was observed with a partial thickness tear of the biceps femoris at the proximal myotendinous junction with surrounding edema without an associated avulsion fracture or hematoma.

Subject #2: Grade 2 strain with partial thickness tear of the left biceps femoris at the mid aspect, extends approximately 6 cm in the craniocaudal dimension.

- Hamstring flexibility and strength

Subject #1: By the third week after the injury, no deficit was evident in hamstring flexibility. By the ninth week, the isometric hamstring strength measurements at 30 and 90 degrees of knee flexion were 92 and 84% than the uninjured side respectively (Fig.1).

Subject #2: Also, by the third week after the injury, no deficit was evident in hamstring flexibility. By the ninth week, the injured side had a higher isometric hamstring strength measurement at 30 and 90 degrees of knee flexion. They were 132% and 110% than the uninjured side respectively (Fig.1).

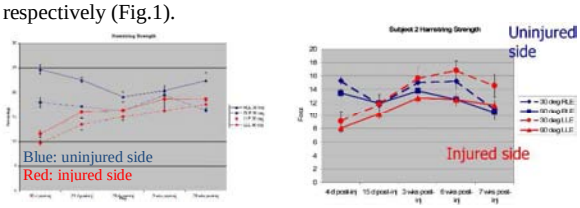


Figure 1: Isometric Hamstring strength (Kg)

- Isokinetic testing results

Subject #1: Eleven weeks after the injury, an isokinetic test of the hamstrings was performed showing an essentially normal result. Peak hamstring torque, on the uninjured side, was 96% of the injured side at 60 degrees per second and 107% of the injured side at 180 degrees per second.

Subject #2: Thirteen weeks after the injury, an isokinetic test of the hamstrings was performed showing an essentially normal result. Peak hamstring torque of the injured side was 96.3% compared to the uninjured side at 60 degrees per second and 97.3% of the uninjured side at 180 degrees per second.

DISCUSSION / CONCLUSION

We have described use of losartan, which is an FDA-approved angiotensin II receptor blocker, to treat two healthy collegiate athletes with a grade 2 biceps femoris injury. The patients tolerated the course of losartan well with no hypotension or any other side effects. Additionally, the patients demonstrated recovery of normal flexibility and strength compared to the contra-lateral leg. Both subjects were ready for return to sports in 9 to 11 weeks after injury.

ACKNOWLEDGEMENTS

This work was supported by the grants of the Henry J. Mankin Endowed Chair in Orthopaedic Surgery at the University of Pittsburgh and by the Albert B. Ferguson, Jr. MD Orthopaedic Fund of The Pittsburgh Foundation.

Improving Recovery Following Recurrent Hamstring Injury Using an Angiotensin II Receptor Blocker: Two Case Studies

INTRODUCTION

Hamstring muscle injuries are common in young competitive athletes. A recent study on National Football League training camp injuries reports hamstring injuries were the most common among muscle strain as well as the most severe. Recurrence is common, secondary to the pressure to return to play prior to complete healing of the injury, with a higher re-injury rate than any other type of injuries. Although muscles can undergo regeneration after injury, the healing process is slow and often culminates in incomplete functional recovery and formation of fibrosis.

A growing understanding of the cellular and molecular events that commonly occur during fibrosis in various tissues, including skeletal muscle, has provided a strong foundation for the development of effective therapies to prevent fibrosis and improve tissue healing. Because TGF-β1 plays such a crucial role in tissue fibrosis, particularly in skeletal muscle, it warrants attention as a key target for anti-fibrotic applications. Of the agents to block TGF-β1, Losartan potassium, a non-peptide molecule that works as an angiotensin II receptor blocker, is particularly attractive for clinical application as it is FDA approved and has minimal side effects. In a murine model, we have found that angiotensin receptor blocker-treated mice exhibited a histological, dose-dependent improvement in muscle regeneration and a significant reduction in fibrous tissue formation within the area of injury.

Given that Losartan has already been used clinically with an extremely safe side effect profile, we have conducted two case studies in young college athletes that sustained recurrent hamstring injuries and whose recoveries were safely improved with losartan. This is an off-label use of losartan (i.e.: the FDA has not approved labeling the device for the described purpose). Here we report the results obtained.

METHODS

Both subjects were submitted to the same protocol that is herein described: after obtaining the subjects' informed consent for treatment, both subjects were started on a 30-day treatment course of losartan at the manufacturer's recommended oral dose of 50 mg per day. Both subjects were healthy and had none of the contra-indications for the use of losartan. In addition to the medication, they underwent a routine rehabilitation program that gradually progressed to eccentric strengthening. The subjects reported no side effects while they were taking the study medication and remained normotensive throughout.

They were initially evaluated by clinical examination and were subsequently evaluated every 7 days with serial measurements (with a hand-held dynamometer - Lafayette Instrument Inc) of hamstring flexibility and strength as well as their blood pressure. Prior to the start of the medication the subjects were submitted to a magnetic resonance imaging (1.5T; GE-Sigma, Waukesha, WI, USA). After a period of 11 weeks the subjects underwent an isokinetic test (Biodex II) to better evaluate muscle strength compared to the non-injured side.

RESULTS

- Subjects

Subject #1: male, 21 years old, college athlete (football punter). He presented 10 days after an acute onset of "searing" pain in his left posterior thigh when he was kicking with his left leg. He referred a similar injury 5 weeks prior to the present injury.

Subject #2: male, 22 years old, college athlete (Ultimate Frisbee). He presented 4 days after an acute onset of pain in his left posterior thigh while he was sprinting. He referred two previous hamstring injuries (2 and 7 months prior to the present injury).

- MRI results (at time of injury)

Subject #1: Acute Grade 2 hamstring strain was observed with a partial thickness tear of the biceps femoris at the proximal myotendinous junction with surrounding edema without an associated avulsion fracture or hematoma.

Subject #2: Grade 2 strain with partial thickness tear of the left biceps femoris at the mid aspect, extends approximately 6 cm in the craniocaudal dimension.

- Hamstring flexibility and strength

Subject #1: By the third week after the injury, no deficit was evident in hamstring flexibility. By the ninth week, the isometric hamstring strength measurements at 30 and 90 degrees of knee flexion were 92 and 84% than the uninjured side respectively (Fig.1).

Subject #2: Also, by the third week after the injury, no deficit was evident in hamstring flexibility. By the ninth week, the injured side had a higher isometric hamstring strength measurement at 30 and 90 degrees of knee flexion. They were 132% and 110% than the uninjured side respectively (Fig.1).

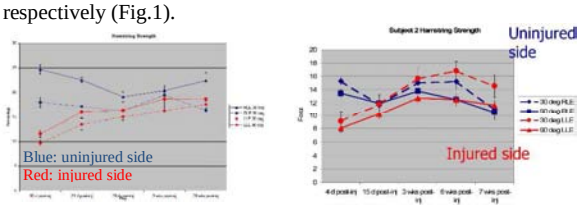


Figure 1: Isometric Hamstring strength (Kg)

- Isokinetic testing results

Subject #1: Eleven weeks after the injury, an isokinetic test of the hamstrings was performed showing an essentially normal result. Peak hamstring torque, on the uninjured side, was 96% of the injured side at 60 degrees per second and 107% of the injured side at 180 degrees per second.

Subject #2: Thirteen weeks after the injury, an isokinetic test of the hamstrings was performed showing an essentially normal result. Peak hamstring torque of the injured side was 96.3% compared to the uninjured side at 60 degrees per second and 97.3% of the uninjured side at 180 degrees per second.

DISCUSSION / CONCLUSION

We have described use of losartan, which is an FDA-approved angiotensin II receptor blocker, to treat two healthy collegiate athletes with a grade 2 biceps femoris injury. The patients tolerated the course of losartan well with no hypotension or any other side effects. Additionally, the patients demonstrated recovery of normal flexibility and strength compared to the contra-lateral leg. Both subjects were ready for return to sports in 9 to 11 weeks after injury.

Follistatin Improves Skeletal Muscle Healing by Blocking TGF-β-like Signaling Pathway

Jinhong Zhu, M.D., Ph.D.,*£ Yong Li, M.D., Ph.D.,*†‡ Aiping Lu, MD.,*† Burhan Gharaibeh, Ph.D.,*† Jianqun Ma, M.D., Ph.D.,*†‡§ Andres J. Quintero, M.D.,* † Tetsuo Kobayashi, M.D.,* † Johnny Huard, Ph.D*†£.

Stem Cell Research Center, Children's Hospital of Pittsburgh, PA,*

Department of Orthopaedic Surgery, University of Pittsburgh, PA, †

Department of Bioengineering, University of Pittsburgh, PA, £

Lab of Molecular Pathology, Department of Pathology, University of Pittsburgh, PA ‡

Division of Thoracic surgery, the Affiliated Tumor Hospital of Harbin Medical University, Harbin, China§

Running head: Follistatin Improves Skeletal Muscle Healing

Number of text page: 34 Number of figures: 7

Funding support

This work was supported by funding from the Henry J. Mankin Endowed Chair for Orthopaedic Research at the University of Pittsburgh, the William F. and Jean W. Donaldson Chair at Children's Hospital of Pittsburgh, the Hirtzel Foundation, and the National Institutes of Health (R01 AR47973 awarded to J.H.).

Corresponding Author:
Dr. Johnny Huard
Henry J. Mankin Professor and Vice Chair for Research
Department of Orthopaedic Surgery,
Professor, Departments of Molecular Genetics and Biochemistry and Bioengineering
Director, Stem Cell Research Center
Children's Hospital of Pittsburgh
4100 Rangos Research Center
3640 Fifth Avenue, Pittsburgh, PA 15213-2582
email: jhuard@pitt.edu; Ph: 412-648-4066; Fax: 412-692-2782

Abstract

Recovery from skeletal muscle injury is often incomplete due to inadequate myofiber regeneration and the formation of fibrosis. Accordingly, injured muscle can benefit significantly from therapies that stimulate muscle regeneration and inhibit fibrosis. To this end, we have focused on doing so by antagonizing a member of the TGF-β superfamily, myostatin. Myostatin is a pharmaceutical target that can be antagonized by follistatin. In vivo, follistatin over-expressing transgenic mice underwent significantly more myofiber regeneration and less fibrosis compared to wild type (WT) mice, as noted after skeletal muscle injury; this is likely partially because follistatin blocks myostatin activity and enhances vacularization. Additionally, the transplantation of muscle progenitor cells, isolated from follistatin over-expressing mice (n = 7) and WT mice (n = 5), into the skeletal muscle of mdx/SCID mice revealed that the follistatin over-expressing donor cells are significantly superior to WT cells at regenerating skeletal muscle fibers (592.79 ± 155.19 vs. 195.6 ± 65.375; Mean ± SEM; p = 0.023). In vitro, follistatin stimulates myoblasts to express MyoD, Myf5, and myogenin, which are myogenic regulatory factors that promote the myogenic differentiation of myoblasts into myotubes. Furthermore, follistatin induces this enhanced differentiation through the inhibition of myostatin, activin A and transforming growth factor –beta 1, which are negative regulators of myoblast differentiation. Our study suggests that follistatin is a promising agent for improving skeletal muscle healing; prior to its pharmacologic application; however, further investigation on follistatin is warranted.

Introduction

Although skeletal muscle injuries are extremely common, accounting for up to 35% to 55% of all sports-related injuries, the treatments that are currently available have not progressed over the last few decades and are often ineffective. Unfortunately, there is significant morbidity associated with these injuries, such as the development of painful contractures, loss of muscle extensibility and strength, and the increased risk for repeat injury, by and large as a result of extensive fibrosis that results from the local secretion of the potent fibrotic cytokine, transforming growth factor – beta 1 (TGF-β1) [1-8].

Our group has identified various anti-fibrotic agents which significantly enhance skeletal muscle healing after injury by antagonizing TGF-β1. These include suramin [9-10], γ-interferon [11], decorin [5, 12-13], relaxin [14-15], and losartan [16]. Although much of the pathogenesis following skeletal muscle injury has been attributed to TGF-β1, we have recently reported that fibrosis is also caused by another agent that limits skeletal muscle healing, namely myostatin [17]. Myostatin is a primary negative regulator for the growth and development of fetal and postnatal skeletal muscle [18-19]. Several groups, including ours, have found that myostatin stimulates fibrosis in injured and diseased skeletal muscle [17, 20-22]. This is because the cell surface receptor to which myostatin predominantly binds -- activin type IIB receptor (ACVR2B) -- activates a downstream TGF-β-like (SMAD2/3) signaling pathway [23-25]. More interestingly, we find the reciprocity between myostatin and TGF-β1: TGF-β1 and myostatin reciprocally induce the expression of one another; blocking TGF-β1 signaling impairs myostatin's biological activity, and vice versa [17]. It suggests that TGF-β1 appears to act in synergy with myostatin to induce fibrosis in the skeletal muscle.

In addition to impairing skeletal muscle healing by promoting fibrosis, myostatin also inhibits myofiber regeneration in diseases such as Duchene's Muscular Dystrophy (DMD) [21] as well as after injury [17, 20]. Specifically mdx mice, an animal model of DMD, were noted to undergo significantly more myofiber damage and less myofiber regeneration along the diaphragm

when compared to myostatin knockout mdx mice [21]. Similarly, following notexin-injury of the tibialis anterior muscle and laceration of the gastrocnemius muscle, there was a significantly greater regeneration and significantly less fibrosis in the myostatin knockout mice when compared to wild type controls [17, 20]. It is interesting to note that current research on muscle regeneration has identified another protein which, much as occurs with myostatin, inhibits myofiber regeneration [26-27]. This protein, namely acitvin A, binds to the same cell surface receptor as myostatin. Accordingly, both of these proteins may be good targets for the development of therapeutic strategies to enhancing skeletal muscle healing.

Research into the development of therapies to antagonize myostatin has led to the discovery of a glycoprotein that neutralizes several proteins of the TGF-β family, namely follistatin. Follistatin has been found to antagonize both myostatin in skeletal muscle and activin A in reproductive tissues, as well as neutralize several other proteins within the TGF-β family [28-31]. Several in vivo studies on follistatin have further shown that the systemic administration of this agent directly inhibits myostatin and also reduces myostatin -induced muscle wasting [19, 30, 32], while follistatin over-expressing transgenic mice exhibit a dramatic increase in muscle mass, much as is seen to occur in myostatin knockout mice [24]. It remains to be determined whether follistatin also promotes skeletal muscle healing by inhibiting myostatin and activin A.

In this report, we provide in vivo and in vitro data to support the development of follistatin as a therapeutic agent for skeletal muscle injury and disease. Specifically, we hypothesize that follistatin over-expressing transgenic mice will undergo more skeletal muscle regeneration and develop less fibrosis after laceration of the gastrocnemius muscles compared to wild type controls. If skeletal muscle healing is superior in the follistatin over-expressing mice compared to wild type controls, then we hypothesize that the muscle progenitor cells of the follistatin over-expressing muscle will outperform cells of wild type muscle, which is likely responsible for this enhanced healing. We also hypothesize that myostatin, acitivin A, and TGF-β1 inhibit myogenic

differentiation of myoblasts in a way that can be rescued by the exogenous addition of follistatin. Finally, we performed experiments to determine how follistatin impacts the expression and downstream signaling of TGF-β1, as well as the expression of myogenic transcription factors, as this likely relates to its impact on skeletal muscle healing.

Materials and methods

Animal model

In accordance with our IACUC approval by the Children's Hospital of Pittsburgh, we performed lacerations according to a previously published protocol [10-13, 15, 17] along the gastrocnemius muscles of 23 male C57BL/6 wild-type (Jackson Laboratories, Bar Harbor, ME) and 23 male follistatin over-expressing mice, each at 7-8 weeks of age, and harvested these muscles at 1 (n = 3), 2 (n = 8), 4 (n = 8) weeks and 1.5 years (n = 4) after laceration injury. From each harvested muscles, we quantified the percentage of fibrosis and muscle regeneration, as previously described [17]. Briefly, following cryosectioning of these tissues histological staining was performed with Masson's trichrome kit (IMEB Inc., Chicago. IL). For each muscle sample, we measured fibrosis from three representative and non-adjacent sections. Briefly, images (200x) were taken up to 3 microscopic fields, and pieced together in Adobe® Photoshop® CS3 to make sure the entire muscle section was covered. We then quantified the percentage of fibrosis with Northern Eclipse software (Empix Imaging, Inc., Cheektawaga, NY), which measures the area of fibrotic tissue along the sites of injury and divide this by the cross-sectional area of tissue to calculate a percentage.

In order to evaluate skeletal muscle regeneration, we stained sections from each harvested muscle with Hematoxylin and Eosin (H&E). For each sample, three non-consecutive sections were chosen; in each section, images (200x) were taken from 2 to 5 microscopic fields, and then pieced together as described above to make sure the entire injured area was covered. The

smallest diameters of centro-nucleated myofibers, which represent regenerating muscle fibers, were quantified with Northern Eclipse software. We measured the diameters of over 350 non-consecutive centro-nucleated myofibers for each gastrocnemius muscles.

Additionally, we performed immunohistochemsitry to detect myostatin (red fluorescence) and collagen type IV (green fluorescence) expression along each injured gastrocnemius muscle. We also stained CD31, an endothelial cell marker, via immunohistostaining to monitor angiogenesis in the injured muscles, and manually counted CD31-positive microvessels with Northern Eclipse software.

Fluorescence Immunostaining

Frozen gastrocnemius muscles were sectioned at 10 μm thickness and immunohistochemical analysis was performed to detect myostatin. Tissue sections were fixed in 4% formalin for 5 minutes followed by two 10-minute washes with PBS. The sections were first incubated with 10% HS for 1 hour to block nonspecific staining. Goat anti-myostatin (R&D Systems, Minneapolis, MN) primary antibody was diluted 1:100 in 2% HS and incubated with sections overnight at 4ºC. Sections were then washed three times with PBS and incubated with a secondary antibody, anti-goat IgG conjugated with biotin (1:200) (Vector Laboratories, Burlingame, CA), for 1 hour at room temperature (RT), followed by a PBS wash. Finally, streptavidin conjugated with Alexa Fluor® 555 (1:500) (Invitrogen, Carlsbad, CA) was applied to each section for an additional hour. DAPI dihydrochloride (4', 6-Diamidino-2-phenylindole dihydrochloride; Sigma, St Louis, MO) was used to counterstain the nuclei. We then quantified the amount of myostatin expression that was present in the injured muscles using Northern Eclipse software, which measures the area and intensity of myostatin signal along the sites of injury. This was then divided by the total cross-sectional area of tissue to calculate the percentage of the injured muscle tissue that is positive for

myostatin. The negative control was stained using the same procedure but without the primary antibody.

To monitor angiogenesis, we first incubated the sections with 10% HS for 1 hour, and then incubated with a rat CD31 primary antibody (BD Biosciences, San Jose, CA) that was diluted 1:150 in 2% HS. This preparation was incubated for 1 hour at RT. The sections were then washed three times with PBS and incubated with the secondary antibody, rabbit anti-rat IgG conjugated with Alexa Fluor® 555 (Invitrogen), for 30 minutes. Finally, DAPI was used to stain the nuclei.

Muscle progenitor cell isolation and transplantation into skeletal muscle: Using a modified preplate technique [33-34], we isolated a fraction of muscle progenitor cells with properties of low adhesion to collagen and long-term proliferation. Briefly, with an anatomical sex technique, we determined the gender of neonatal mice. With these methods combined, we isolated five populations of wild type muscle progenitor cells from 5 male neonatal C57BL/6J mice, as well as 7 populations of follistatin over-expressing muscle progenitor cells from 7 male neonatal follistatin over-expressing mice with a background of C57BL/6J mice. These cells were expanded in proliferation medium consisting of Dulbecco's modified Eagle's medium (DMEM; Invitrogen), 10% horse serum (Invitrogen), 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin/streptomycin (P/S; Invitrogen), and 0.5% chicken embryo extract (Accurate Chemical & Scientific Corporation, Westbury, NY).

Flow Cytometry

In order to characterize wild type and follistatin over-expressing muscle progenitor cell populations, we utilized flow cytometry specific to the cell markers CD34, Sca-1 in wild type and follistatin over-expressing muscle progenitor cells to analyze the percentage of stem cells in the muscle progenitor cell populations as previously described [33-34]. Briefly, cultured cells were trypsinized, centrifuged, and washed twice with PBS. We subsequently re-suspended our cell

pellets, blocked them with 10% mouse serum (Sigma) for 10 minutes on ice and applied rat anti-mouse monoclonal conjugated antibodies (CD34-PE, Sca-1-APC; BD Biosciences) to incubate on ice for 30 minutes. Following this incubation period, we excluded nonviable cells by adding 7-amino-actinomycin D (7-AAD; BD Biosciences) to each sample. Cells were then evaluated with a FACS Caliber flow cytometer (Becton Dickinson) and analyzed with CellQuest software (Becton Dickinson).

After isolating muscle progenitor cells, these cells were injected into the gastrocnemius muscles of female mdx/SCID mice; these mice were bred by crossing mdx (C57BL/10ScSn-Dmdmdx) and SCID (C57BL/6J-prkdcscid/SzJ) mice (Jackson laboratory) at our institution's animal facility. Approximately 3×10^5 cells from each cell population were transplanted in each gastrocnemius muscles, for a total of 4 gastrocnemius muscles among recipient female mdx/SCID mice. All mdx/SCID mice used were littermates, and all were sacrificed 2 weeks post-transplantation. The recipient gastrocnemius muscles from these mice were harvested at this time, snap-frozen, and cryosectioned on a later date at thicknesses of 10 μm.

Each section was immunostained for dystrophin with a rabbit anti-mouse dystrophin antibody in order to monitor the number of dystrophin positive myofibers formed by the donor muscle progenitor cells. These tissue sections were then fixed in 5 % formalin for 5 minutes, followed by two 10-minute washes with PBS. These sections were blocked with 10% donkey serum for 1 hour, and sections were then incubated overnight at 4oC in a rabbit dystrophin primary antibody that was diluted 1:500 in 5% donkey serum. The following day, these sections were washed three times with PBS and incubated with the secondary antibody, donkey anti-rabbit IgG conjugated with Alexa Fluor® 594 (Invitrogen). Images (200x) of up to 10 microscopic fields of the sections, representing the largest dystrophin-positive engraftment areas, were taken for each sample. Images were put together to in order to cover the entire engraftment area as shown in Figure 5. C.

Dystrophin-positive myofibers were then counted to assess the efficiency of cell transplantation in the skeletal muscle of mdx/SCID mice.

Cell culture

A C2C12 myoblast cell line (ATCC, Manassas, VA) was seeded overnight onto collagen-coated 12-well plates in normal growth medium (10% FBS and 1% PS in DMEM). The next day, this medium was replaced with low-serum medium (2% horse serum and 1% P/S in DMEM), followed by medium and recombinant protein changes every 48 hours.

In the first set of experiments, C2C12 myoblasts were stimulated with follistatin recombinant protein (Sigma, St. Louis, MO) and cultured up to 4 days. The effect of follistatin on myogenic differentiation and expression of MyoD, Myf5, myogenin, and myostatin were examined by myosin heavy chain (MyHC) immunostaining and western blot analysis.

In the second set of experiments, C2C12 myoblasts were stimulated with a combination of follistatin, Activin A, and myostatin. The myogenic differentiation of these cells was then evaluated via MyHC immunostaining by calculating the fusion index (i.e., the ratio of nuclei in myotubes to all nuclei) according to previously published protocol [17]. Images of 5 representative microscopic fields were taken of each well from the 12-well plates.

In the final set of experiments, we incubated C2C12 myoblasts with varying concentrations of recombinant proteins (ie., follistatin, TGF-β1). From these cells, those that were used to stain for myogenic differentiation with MyHC immunostaining were cultured for 4 days prior to staining, while the other cells were incubated for 16 hours prior to detecting TGF-β1, SMAD2, and P-SMAD2. In the latter set of cells, cell lysates were collected in preparation for western blot assays; SDS-PAGE was used to separate proteins, and specific antibodies were applied to detect target proteins.

Western Blot

Cultured cells were lysed with T-PER® Tissue Protein Extraction Reagent with the addition of protease inhibitors (Pierce, Rockford, IL). Equal amounts of cellular protein were loaded into each well and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Nitrocelluose membrane blotting was then performed under standard conditions. For immnobloting, we used the following primary antibodies: mouse anti-β-actin IgG (1:8,000) (Sigma), rabbit anti-myostatin IgG (1:3,000) (R&D Systems), rabbit anti-TGF-β1 IgG (1:1000) (Abcam Inc. Cambridge, MA), goat anti-Myf-5 (Santa Cruz Biotechnology, Inc.), mouse anti-MyoD, myogenin IgGs (1:250) (BD Biosciences), goat anti-SMAD2, p-SMAD2 IgGs (Cell signaling Technology, Danvers, MA).

Statistics

All data are reported as the mean ± standard deviation (SD) or mean ± standard error of the mean (SEM), and data analyses have been performed with Student's t-test for comparisons between two groups as well as with a One-Way ANOVA for comparisons among three or more groups (SPSS Jandel Corporation). For all statistically significant differences observed after a One-Way ANOVA, the appropriate multiple comparison tests have been used to perform a post-hoc analysis. Statistical significance is considered for all p-values < 0.05; values marked with asterisks (*) and (**) represent $P < 0.05$, $P < 0.01$, respectively.

Results

Healing after injury is enhanced in follistatin over-expressing skeletal muscle. Following laceration, the gastrocnemius muscles from wild type (WT) and follistatin over-expressing (FLST/OE) mice regenerated as confirmed by visualizing centro-nucleated myofibers and variability of fiber size (i.e., fiber diameter) (Fig. 1A). The myofiber diameters were determined 7

days after laceration and they ranged in size from 5-35 μm. (Fig. 1B). Over time, the diameters of regenerating myofibers increased in size with the mean diameter of the follistatin over-expressing regenerating myofibers being consistently and significantly larger than that of the wild type regenerating muscle fibers. The mean regenerating fiber diameters for follistatin over-expressing mice as measured at post-laceration 7, 14, 30 days, and 1.5 years were larger by approximately 25.3% (19.37 ± 0.80 vs. 15.46 ± 0.81; $p < 0.01$**), 31.6% (39.77 ± 3.69 vs. 30.22 ± 2.75; $p < 0.01$**), 32.5% (45.55 ± 3.03 vs. 34.38 ± 1.56; $p < 0.01$**), and 36.3% (64.36 ± 5.4 vs. 47.22 ± 3.49; $p < 0.01$**) respectively, than those of wild type mice (Fig. 1C). The absolute differences in mean diameters of regenerating myofibers between wild type and follistatin over-expressing mice were also increasing (green curve Fig. 1C). Accordingly, healing muscles of follistatin over-expressing mice compared to wild type animals contain a larger percentage of larger myofibers at each time point. For example, at 7 days 51% of regenerating wild type myofibers were larger than 15 μm, whereas, 86% of regenerating follistatin over-expressing myofibers were larger than 15 μm; at 14 days, 45% of regenerating myofibers of wild type mice were larger than 30 μm. In contrast, 73% of regenerating myofibers of follistatin over-expressing mice fall in to the category of 30 to 85 μm; at 30 days, 40% of regenerating myofibers of wilde type mice were larger than 35 μm, while 73% of regenerating follistatin over-expressing myofibers were larger than 35 μm (Fig. 1B).

In addition to noticing differences in myofiber regeneration, we observed significant differences in the deposition of collagenous connective tissue after injury between wild type and follistatin over-expressing muscles. Specifically at 14 days after laceration, fibrosis developed extensively in wild type muscles, but relatively limited in follistatin over-expressing muscles (Fig. 2A). The percentage of fibrosis quantified at 14 days post-injury within wild type and follistatin over-expressing gastrocnemius muscles, respectively, was 8.71% ± 2.36% and 3.54% ± 1.71% ($p < 0.01$**) (Fig. 2B). Compared to these values obtained at 14 days, our quantification analysis showed a reduction in fibrosis at 30 days among gastrocnemius muscles of wild type and

follistatin over-expressing mice (Fig. 2A); in spite of this, the relative fibrosis formation in wild type gastrocnemius muscles continued to be significantly larger than follistatin over-expressing gastrocnemius muscles (5.57 ± 1.94 vs. 2.1 ± 1.1; $P < 0.01$**) at 30 days post-injury (Fig. 2B). However, fibrosis in both injured gastronemius muscles of wild type and follistatin over-expressing mice disappeared at 1.5 years after injury (data not shown).

Upon investigating how follistatin enhances muscle healing, we found that, in vivo, apart from reported mechanisms that follistatin's inhibition of myostatin via direct binding [24, 30], it also represses myostatin expression in injured follistatin over-expressing muscle. This became apparent at two weeks after injury, when we noted that follistatin over-expressing muscle expresses less myostatin compared to wild type muscle (Fig. 3A, B). Collagen IV (green) immunostaining, stains the basal lamina, which is seen to outline the myofibers. Myostatin protein (red) mostly locates in the cytoplasm of regenerating fibers as indicated by collagen IV and Centro-nuclei (Fig. 3A arrows). However, some of myostatin-positive regenerating myofibers lacked the complete basal lamina (Fig. 3A arrowheads). When measuring the area and intensity of myostatin signal in the injured muscles, we found that myostatin in the injured follistatin over-expressing muscle was significantly lower than observed in the wild type controls. We also found a trend that phosphorylation level of SMAD2/3 in the injured follistatin over-exrpessing muscles is lower than that in the wild type control (supplemental data). Moreover, it has been found that follistatin stimulates angiogenesis both in vitro and in vivo [35], and a negative correlation has been reported between angiogenesis and fibrosis [36]. These findings lead us to examine vascularization in the injured follistatin over-expressing and wild type muscles. We assessed the vascularity of injured follistatin over-expressing and wild type muscles at 30 days following laceration, and observed that follistatin over-expressing muscles have a significantly larger number of CD31-positive capillary-like structures along the zone of injury in comparison to their wild type

counterparts (Fig. 3C, D). This indicates that increased vascularity may at least be partially responsible for the improved muscle healing that is observed in follistatin over-expressing mice.

Comparison of Wild Type- and Follistatin Over-expressing-Muscle Progenitor Cells

To further characterize wild type- and follistatin over-expressing muscle progenitor cells (FLST/OE-MPCs), we used flow cytometry and immnocytochemistry to analyze the expressions of stem cell markers (i.e., Sca-1, CD34) and myogenic (Desmin) markers by these cells, and also determined proliferation and myogenic differentiation capacity of cell populations. From the histograms (Sca-1+&CD34+, Sca-1+, and CD34+) in Fig. 4A, the heterogeneous profile of stem cell markers is apparent; cell populations within either the follistatin over-expressing or wild type groups exhibited great variation in the percentages of stem cell marker positive cells. Compared to wild type- muscle progenitor cell populations, follistatin over-expressing populations possessed a significantly larger percentage of cells that were positive for Sca-1 (Fig. 4A, B). The representative images of the flow dot plot showed that one follistatin over-expressing muscle progenitor cell and one wild type muscle progenitor cell population contained 46.5% and 24% Sca-1 positive cells, respectively (Fig. 4C). In the low serum medium, both follistatin over-expressing and wild type muscle progenitor cells were capable of fusing into multinucleated myotubes as labeled by DAPI and myosin heavy chain (MyHC) (Fig. 4D). The percentage of desmin-positive cells, proliferation, and myogenic differentiation capacity were compared between the follistatin over-expressing and wild type- muscle progenitor cell populations; however, due to a broad variability, no significant difference was found (data not shown).

Muscle progenitor cells are a population of primary long-term proliferating cells that regenerate the skeletal muscle more efficiently than myoblasts when transplanted into dystrophic

mice [34, 37]. Since injured follistatin over-expressing muscles underwent better muscle regeneration than did wild type controls, follistatin over-expressing muscle progenitor cells may accordingly be superior to wild type- muscle progenitor cells in regenerating muscle. Using a preplate technique that our group has previously described [34, 37], we isolated these cells from both wild type mice and follistatin over-expressing mice and compared their ability to regenerate skeletal muscle in dystrophic mice. Wild type- and follistatin over-expressing muscle progenitor cell populations were injected into the gastrocnemius muscles of mdx/SCID mice. Quantitation of engraftment with regard to the number of dystrophin-positive myofibers was performed to evaluate the cell transplantation efficiency. Notwithstanding a high degree of variability in the wild type- and follistatin over-expressing muscle progenitor cells' abilities to regenerate myofibers in vivo (Fig. 5A), only 2 out of 5 wild type- muscle progenitor cell populations produced over 200 dystrophin-positive myofibers; in comparison, 6 out of 7 follistatin over-expressing populations regenerated more than 200 myofibers. Specifically, whereas the wild type- muscle progenitor cell population with the greatest amount of dystrophin-positive fiber regenerating was able to regenerate 400 fibers, 4 of 7 follistatin over-expressing populations regenerated between 30 and 1398 fibers (Fig. 5A). Moreover, all the follistatin over-expressing muscle progenitor cells produced more fibers than the mean fibers (e.g., 195.6 myofibers) produced from wild type- muscle progenitor cells populations (Fig. 4A). Overall, follistatin over-expressing muscle progenitor cells (n = 7) produced significantly larger muscle engraftment than did wild type cells (n = 5) (592.79 ± 154.9 vs. 195.6 ± 65.375; Mean ± SEM; P = 0.023*; t-test) (Fig. 5B). Two representative dystrophin-positive engraftment-derived from follistatin over-expressing and wild type muscle progenitor cells (784 vs. 494 dystrophin-positive myofibers), respectively, are shown in Fig 5C.

Interaction between Follistatin and TGF-βs

In order to investigate how follistatin interferes with myostatin function, we found that in vitro, myostatin inhibits myogenic differentiation of C2C12 myoblasts, nevertheless, follistatin neutralizes exogenous myostatin, and thereby permits myoblasts to undergo myogenic differentiation (Fig. 6A). We further showed that, much as is the case with myostatin and myoblasts, activin A also significantly inhibits the myogenic differentiation of C2C12 cells (Fig. 6B), and that supplementing cell cultures with follistatin also significantly attenuates the inhibitory effect of activin A on C2C12 myoblasts's capacity to differentiate into myotubes (Fig. 6C).

TGF-β1 can exert both a positive and negative effects on cellular proliferation and differentiation, as the biological actions of TGF-β1 on cell behaviors are highly cell type specific. For instance, TGF-β1 stimulates endothelial cell differentiation, whereas it inhibits adipogenic and myogenic differentiation [38-39]. Myogenic differentiation in particular involves sequential steps that myoblasts initially retract from the cell cycle and thereafter differentiate and fuse into multi-nucleated myotubes. Although muscle cells are induced to withdraw from the cell cycles following cell culturing in TGF-β1-containing and fusion-promoting media, these cells nonetheless fail to fuse into myotubes, as is evident by the lack of muscle creatine kinase and nicotinic acetylcholine receptor expression [40]. This is consistent with our experiments, which show that TGF-β1 inhibits the myogenic differentiation of C2C12 myoblasts. Surprisingly, follistatin was able to reverse this process, permitting these cells to proceed toward myogenic differentiation (Fig. 7A, B). From representative image, we were able to see plenty of myosin heavy chain-positive myotubes in the control cell culture (i.e., without treatment). With follistatin treatment, myotubes further fuse to form larger myotubes (Fig. 7A, B). In opposition to what observed in control, very few smaller myotubes interspersed among numerous nuclei (blue) in cell cultures treated with TGF-β1. In cells treated with both TGF-β1 and increasing

concentrations of follistatin, follistatin seemed to counteract TGF-β1's inhibition on myogenesis of C2C12 myoblasts witnessed by the formation of larger myotubes than those seen in the TGF-β1 only treated group (Fig. 7A). The fusion index indicated that follistatin significantly increased myogenic differentiation and that TGF-β1 significantly decreased myogenic differentiation; whereas, follistatin prevented TGF-β1 from inhibiting myogenesis, partially restoring myogenic differentiation of C2C12 myoblasts (Fig. 7B). Follow-up experiments indicated that follistatin decreases TGF-β1 expression in C2C12 myoblasts, as noted both with and without the exogenous application of TGF-β1 (Fig 7C). Additionally, the TGF-β1 signaling pathway relies on the activation of an intracellular SMAD signaling cascade, and our results indicate that follistatin blocks this pathway by reducing the expression and phosphorylation of SMAD2 (Fig 7D).

From prior studies, our group has shown that follistatin stimulates C2C12 myoblasts to undergo myogenic differentiation [17]. Follistatin significantly increases the fusion index of the cells when compared to controls, and does so in a dose-dependent fashion [17]. In our complementary experiments from the current study, in follistatin-treated C2C12 cells, the early stage of myogenic differentiation--during which there is no detectable formation of myotubes--there is a notable increase in the expressions of MyoD and Myf5 compared to cells without follistatin treatment. During the late stages of differentiation, in which the formation of myotubes is quite extensive, C2C12 cells exposed to follistatin augment their expression of myogenin and maintain elevated expression levels of Myf-5 compared to controls (Fig. 7E). Finally, aside from directly neutralizing myostatin, follistatin also appears to reduce the expression of myostatin by C2C12 cells at the early stage of myogenic differentiation (Fig. 7E, arrowheads).

Discussion

In this study, we show that the skeletal muscle healing of follistatin over-expressing mice is superior to that of wild type mice. Specifically, the mean diameter of regenerating myofibers in

injured follistatin over-expressing muscle remain significantly larger than wild type counterparts, while fibrosis was significantly lower in the former; these results are comparable to those that we previously obtained from injured myostatin knockout gastrocnemius muscles [17]. There are several possible explanations for this, which were suggested by our results. These include the impacts that follistatin has on a) down-regulating the expression level of myostatin and phosphrylation of Smad2, b) augmenting vascularity in the injured muscles, c) enhancing the ability of muscle progenitor cells to regenerate skeletal muscles. The last one was confirmed by in vitro results showing that follistatin promotes myoblast differentiation by blocking the negative regulators, myostatin, activin A, and TGF-β1, and augments the expression of various myogenic factors by myoblast. Although we will discuss these events individually, we highlight that they are not mutually exclusive of one another, but rather, illustrate how follistatin can synergistically promote healing through each of these processes.

Mechanisms Involved in the Reduced Fibrosis in the Injured Follistatin Over-expressing Muscle

TGF-β1 expression in injured skeletal muscle is time dependent; it peaks at 3-5 days and 10-14 days post-injury [5-6, 41]. The latter event appears to associate with the formation of fibrosis and ineffective muscle regeneration [5]. Blocking the second peak of TGF-β1 by administrating an antifibrotic agent at 14 days post-injury led to histological and physiological improvements of the injured muscles [5, 9-15]. Coincidently, our in vivo studies showed significant decreases in myostatin immunostaining among injured follistatin over-expressing gastrocnemius muscles at 14 days after injury. This result indicates that, in addition to directly inhibiting myostatin activity, follistatin may further decrease the expression of this protein. Similar to our previous findings [17], some small regenerating myofibers without basal lamina were strongly myostatin positive in the injured wild type and follistatin over-expressing gastrocnemius muscles. Li et al reported that

some regenerating myofibers appeared to degrade and transform into myofibroblasts to aggravate fibrosis in the injured skeletal muscles [5]. If this is the case, these myostatin positive, basal lamina deficient regenerating fibers may represent a transitional state of regenerating myofibers that are undergoing the differentiation process into myofibroblasts. The decrease in the amount of myostatin at the injury site probably partially accounts for the reduced fibrosis in injured follistatin over-expressing muscle for following reasons: a) there is less myostatin available to trigger fibrosis in injured muscle, b) due to the fact that TGF-β1and myostatin synergistically induce fibrosis in the injured skeletal muscles [17], reduced amount myostatin may compromise TGF-β1's pro-fibrotic effect. Moreover, we show that follistatin down-regulates the expression of TGF-β1 and counteracts its activity in vitro. Myostatin, TGF-β1, and activin A all belong to the TGF-β superfamily which all signal through the TGF-β/SMAD2/3 signaling pathway. Athough we don't have direct evidence showing, that follistatin may also reduce fibrosis through the inhibition of TGF-β1 and activin A, our data indicates that there is an overall decrease in the phosphorylation level of SMAD2/3 of the injured follistatin over-expressing muscle when compared to the wild type control. Taken together, our results appear to indicate that follistatin decreases the TGF-β-like signaling that occurs through the SMAD2/3 pathway, thereby attenuating the inhibitory effect of this pathway on skeletal muscle healing.

Furthermore, while prior reports indicate that follistatin up-regulates angiogenesis both in vivo and in vitro [35, 42-43], we show that there is significantly more CD-31 positive capillary-like structures occurring in injured follistatin over-expressing muscle than in wild type controls. Activin A inhibited growth and activation of vascular endothelial cells [42, 44]. Endothelial cells express both activin A and follistatin. Activin A is constitutively expressed in endothelial cells; however, the expression of follistatin is subject to dynamic regulation [42-43]. For instance, follistatin can be expressed by endothelial cells that are activated, but down-regulated in the quiescent cells [42]. It is thereby also noteworthy to point out that angiogenesis correlates with an increase in skeletal and cardiac muscle regeneration and a decrease in fibrosis [36, 45-47]. Presently,

however, there is insufficient evidence to indicate whether follistatin directly stimulates angiogenesis in injured skeletal muscle. In the current study, we showed that in the absence of myostatin signal, CD31-positive structures were increased in the healing skeletal muscle. However, more evidence is needed to prove these CD31-positive structures can function like blood vessels.

Cellular Mechanism by Which Follistatin Promotes Skeletal Muscle Regeneration after Injury.

The development of skeletal muscle during embryogenesis and its regeneration after trauma or in the setting of skeletal muscle disease largely occurs from the differentiation of satellite cells into myofibers [48-50]. Adult satellite cells enter cellular quiescence within a niche between the basal lamina and sarcolemma of the myofibers, thereby forming a pool of myogenic progenitor cells [51]. In response to muscle trauma and during disease, these cells are activated to re-enter the cell cycle, migrate from the basal lamina to the zone of injury, and undergo asymmetric divisions. A preponderance of daughter cells are committed to differentiate and fuse into multinucleated myofibers, while a small portion of self-renewing cells replenish the reservoir of satellite cells by re-entering quiescence.

Myostatin inhibits satellite cell self-renewal by down-regulating the G1 to S progression and retaining satellite cells in a quiescent status [52-53]; inversely, myostatin knockout skeletal muscle possesses more satellite cells than their wild type counterparts, likely resulting from an increase in proliferation and a delay in myogenic differentiation by the adult myostatin knockout satellite cells [52]. Based on this information, we sought to determine the impact that follistatin would have on muscle progenitor cells. Muscle progenitor cells are a heterogeneous population consisting of myoblasts, satellite cells, progenitor cells, and stem cells. We isolated muscle progenitor cells from the gastrocnemius muscle of both follistatin over-expressing and wild type mice using the

modified pre-plate technique as described previously [54]. We next injected these cell populations into the gastrocnemius muscles of mdx/SCID mice to determine their regeneration efficiency. We found that overall, follistatin over-expressing muscle progenitor cell populations regenerated significantly more dystrophin-positive myofibers; however, it should be noted that not all the follistatin over-expressing muscle progenitor cell populations outperformed their wild type counterparts. Variations in regard to the regenerative capacity of both cell types was readily apparent and is a common phenomenon seen in myoblasts, satellite cells, progenitor cells, and muscle stem cells collectively [55]. Specifically, dystrophin-positive myofibers regenerated from follistatin over-expressing muscle progenitor cells populations varied from 200 to 1398 fibers, while those produced from wild type- muscle progenitor cells populations varied from 27 to 400 fibers. The increase in regenerative capacity of follistatin over-expressing muscle progenitor cells populations probably partially accounts for the better regeneration in the injured follistatin over-expressing muscle than is seen in wild type muscle. These findings may help researchers to genetically engineer an optimal cell population for cell-based therapy to treat Duchene Muscular Dystrophy (DMD), a lethal sex-linked recessive, muscle-wasting disease which stems from a variety of different mutations of the dystrophin gene [56-57]. For instance, the transplantation of muscle cells carrying a follistatin transgene into the dystrophic muscle of a DMD patient may enhance the success of cell transplantation in comparison to that of unengineered muscle progenitor cells [58].

We also investigated whether the cell surface marker profile, proliferation rate, and myogenic differentiation capacity of muscle progenitor cells could render a clue to their in vivo performance. Our group previously found that the regenerative capacity of muscle cells appears to be negatively related to the level of their in vitro myogenic commitments when they compared different fractions among the same muscle primary cell population [59]. Moreover, they further showed that the CD34+ fraction of muscle progenitor cells showed significant improvements in dystrophin restoration and myotube regeneration after being transplanted into dystrophic muscle,

when compared to the CD34- fraction of the same muscle progenitor cell population [59]. Nevertheless, these criteria can not be applied to this current study, since we are comparing cell performances among muscle progenitor cell populations isolated from different mice. We instead found that muscle progenitor cell populations isolated from both types of mice exhibited a broad heterogeneity of cell surface markers, proliferation rate, and myogenic capacity in vitro. In spite of this heterogeneity, the follistatin over-expressing muscle progenitor cells populations contain a significantly larger percentage of Sca-1+ cells than the wild type controls. It was reported that among male muscle-derived stem cell populations, a higher percentage of Sca-1+ cells in the cell populations appeared to positively correlate with a higher regenerative capacity in the recipient dystrophic muscle; however, this is not the case for female muscle-derived stem cells [55]. These findings are consistent with ours, since our entire muscle progenitor cell populations were isolated from male mice.

Collectively, our results suggest that the degree of in vitro myogenic commitment, proliferation rate, and expression levels of stem cell and myogenic markers cannot be used to predict the cell's in vivo performance among the different cell populations, which is consistent with data collected by others in our group [55]. The potential predictor of the cells' regeneration capacity requires additional exploration.

Molecular mechanisms by which Follistatin Promotes Myoblast Differentiation

As stated above, we found that the differentiation of myoblasts in vitro is inhibited by myostatin, activin A, and TGF-β1. Each of these factors appears to be inhibited by follistatin, as a result, myoblast differentiation capacity can be restored. While this occurs via direct antagonism of myostatin and activin A, respectively, it is currently unclear whether follistatin inhibits TGF-β1 directly or indirectly.

We conclude that follistatin has great therapeutic potential for the treatment of injured skeletal muscles, as it enhances the skeletal muscle healing. This is illustrated through experiments in which there is an increase in skeletal muscle regeneration and a decrease in fibrosis along the injury zone of follistatin over-expressing mice, as well as in experiments where myostatin expression and phosphylation levels of SMAD2/3 are down-regulated in follistatin over-expressing muscles after injury. As expected from these findings, we show that injured follistatin over-expressing muscles undergo significantly more vascularization compared to controls, and that follistatin over-expressing muscle progenitor cells have a superior regenerative capacity compared to wild type- muscle progenitor cells . Moreover, we found that follistatin promoted myogenesis by inhibiting activin A and TGF-β1, two potent fibrotic agents that otherwise inhibit efficient regeneration.

References

1. Border WA, Noble NA: Transforming growth factor beta in tissue fibrosis, N Engl J Med 1994, 331:1286-1292

2. Lijnen PJ, Petrov VV, Fagard RH: Induction of cardiac fibrosis by transforming growth factor-beta(1), Mol Genet Metab 2000, 71:418-435

3. Waltenberger J, Lundin L, Oberg K, Wilander E, Miyazono K, Heldin CH, Funa K: Involvement of transforming growth factor-beta in the formation of fibrotic lesions in carcinoid heart disease, Am J Pathol 1993, 142:71-78

4. Yamamoto T, Noble NA, Miller DE, Border WA: Sustained expression of TGF-beta 1 underlies development of progressive kidney fibrosis, Kidney Int 1994, 45:916-927

5. Li Y, Foster W, Deasy BM, Chan Y, Prisk V, Tang Y, Cummins J, Huard J: Transforming growth factor-beta1 induces the differentiation of myogenic cells into fibrotic cells in injured skeletal muscle: a key event in muscle fibrogenesis, Am J Pathol 2004, 164:1007-1019

6. Li Y, Huard J: Differentiation of muscle-derived cells into myofibroblasts in injured skeletal muscle, Am J Pathol 2002, 161:895-907

7. Bernasconi P, Torchiana E, Confalonieri P, Brugnoni R, Barresi R, Mora M, Cornelio F, Morandi L, Mantegazza R: Expression of transforming growth factor-beta 1 in dystrophic patient muscles correlates with fibrosis. Pathogenetic role of a fibrogenic cytokine, J Clin Invest 1995, 96:1137-1144

8. Gosselin LE, Williams JE, Deering M, Brazeau D, Koury S, Martinez DA: Localization and early time course of TGF-beta 1 mRNA expression in dystrophic muscle, Muscle Nerve 2004, 30:645-653

9. Chan YS, Li Y, Foster W, Fu FH, Huard J: The use of suramin, an antifibrotic agent, to improve muscle recovery after strain injury, Am J Sports Med 2005, 33:43-51

10. Chan YS, Li Y, Foster W, Horaguchi T, Somogyi G, Fu FH, Huard J: Antifibrotic effects of suramin in injured skeletal muscle after laceration, J Appl Physiol 2003, 95:771-780

11. Foster W, Li Y, Usas A, Somogyi G, Huard J: Gamma interferon as an antifibrosis agent in skeletal muscle, J Orthop Res 2003, 21:798-804

12. Sato K, Li Y, Foster W, Fukushima K, Badlani N, Adachi N, Usas A, Fu FH, Huard J: Improvement of muscle healing through enhancement of muscle regeneration and prevention of fibrosis, Muscle Nerve 2003, 28:365-372

13. Fukushima K, Badlani N, Usas A, Riano F, Fu F, Huard J: The use of an antifibrosis agent to improve muscle recovery after laceration, Am J Sports Med 2001, 29:394-402

14. Li Y, Negishi S, Sakamoto M, Usas A, Huard J: The use of relaxin improves healing in injured muscle, Ann N Y Acad Sci 2005, 1041:395-397

15. Negishi S, Li Y, Usas A, Fu FH, Huard J: The effect of relaxin treatment on skeletal muscle injuries, Am J Sports Med 2005, 33:1816-1824

16. Bedair HS, Karthikeyan T, Quintero A, Li Y, Huard J: Angiotensin II receptor blockade administered after injury improves muscle regeneration and decreases fibrosis in normal skeletal muscle, Am J Sports Med 2008, 36:1548-1554

17. Zhu J, Li Y, Shen W, Qiao C, Ambrosio F, Lavasani M, Nozaki M, Branca MF, Huard J: Relationships between transforming growth factor-beta1, myostatin, and decorin: implications for skeletal muscle fibrosis, J Biol Chem 2007, 282:25852-25863

18. McPherron AC, Lawler AM, Lee SJ: Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member, Nature 1997, 387:83-90

19. Zimmers TA, Davies MV, Koniaris LG, Haynes P, Esquela AF, Tomkinson KN, McPherron AC, Wolfman NM, Lee SJ: Induction of cachexia in mice by systemically administered myostatin, Science 2002, 296:1486-1488

20. McCroskery S, Thomas M, Platt L, Hennebry A, Nishimura T, McLeay L, Sharma M, Kambadur R: Improved muscle healing through enhanced regeneration and reduced fibrosis in myostatin-null mice, J Cell Sci 2005, 118:3531-3541

21. Wagner KR, McPherron AC, Winik N, Lee SJ: Loss of myostatin attenuates severity of muscular dystrophy in mdx mice, Ann Neurol 2002, 52:832-836

22. Li ZB, Kollias HD, Wagner KR: Myostatin directly regulates skeletal muscle fibrosis, J Biol Chem 2008,

23. Zhu X, Topouzis S, Liang LF, Stotish RL: Myostatin signaling through Smad2, Smad3 and Smad4 is regulated by the inhibitory Smad7 by a negative feedback mechanism, Cytokine 2004, 26:262-272

24. Lee SJ, McPherron AC: Regulation of myostatin activity and muscle growth, Proc Natl Acad Sci U S A 2001, 98:9306-9311

25. Rebbapragada A, Benchabane H, Wrana JL, Celeste AJ, Attisano L: Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis, Mol Cell Biol 2003, 23:7230-7242

26. He L, Vichev K, Macharia R, Huang R, Christ B, Patel K, Amthor H: Activin A inhibits formation of skeletal muscle during chick development, Anat Embryol (Berl) 2005, 209:401-407

27. Link BA, Nishi R: Opposing effects of activin A and follistatin on developing skeletal muscle cells, Exp Cell Res 1997, 233:350-362

28. Fainsod A, Deissler K, Yelin R, Marom K, Epstein M, Pillemer G, Steinbeisser H, Blum M: The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4, Mech Dev 1997, 63:39-50

29. Gamer LW, Wolfman NM, Celeste AJ, Hattersley G, Hewick R, Rosen V: A novel BMP expressed in developing mouse limb, spinal cord, and tail bud is a potent mesoderm inducer in Xenopus embryos, Dev Biol 1999, 208:222-232

30. Amthor H, Nicholas G, McKinnell I, Kemp CF, Sharma M, Kambadur R, Patel K: Follistatin complexes Myostatin and antagonises Myostatin-mediated inhibition of myogenesis, Dev Biol 2004, 270:19-30

31. Thompson TB, Lerch TF, Cook RW, Woodruff TK, Jardetzky TS: The structure of the follistatin:activin complex reveals antagonism of both type I and type II receptor binding, Dev Cell 2005, 9:535-543

32. Nakatani M, Takehara Y, Sugino H, Matsumoto M, Hashimoto O, Hasegawa Y, Murakami T, Uezumi A, Takeda S, Noji S, Sunada Y, Tsuchida K: Transgenic expression of a myostatin inhibitor derived from follistatin increases skeletal muscle mass and ameliorates dystrophic pathology in mdx mice, Faseb J 2008, 22:477-487

33. Jankowski RJ, Haluszczak C, Trucco M, Huard J: Flow cytometric characterization of myogenic cell populations obtained via the preplate technique:

potential for rapid isolation of muscle-derived stem cells, Hum Gene Ther 2001, 12:619-628

34. Qu-Petersen Z, Deasy B, Jankowski R, Ikezawa M, Cummins J, Pruchnic R, Mytinger J, Cao B, Gates C, Wernig A, Huard J: Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration, J Cell Biol 2002, 157:851-864

35. Krneta J, Kroll J, Alves F, Prahst C, Sananbenesi F, Dullin C, Kimmina S, Phillips DJ, Augustin HG: Dissociation of angiogenesis and tumorigenesis in follistatin- and activin-expressing tumors, Cancer Res 2006, 66:5686-5695

36. Nguyen F, Guigand L, Goubault-Leroux I, Wyers M, Cherel Y: Microvessel density in muscles of dogs with golden retriever muscular dystrophy, Neuromuscul Disord 2005, 15:154-163

37. Deasy BM, Gharaibeh BM, Pollett JB, Jones MM, Lucas MA, Kanda Y, Huard J: Long-term self-renewal of postnatal muscle-derived stem cells, Mol Biol Cell 2005, 16:3323-3333

38. Ignotz RA, Massague J: Type beta transforming growth factor controls the adipogenic differentiation of 3T3 fibroblasts, Proc Natl Acad Sci U S A 1985, 82:8530-8534

39. Masui T, Wakefield LM, Lechner JF, LaVeck MA, Sporn MB, Harris CC: Type beta transforming growth factor is the primary differentiation-inducing serum factor for normal human bronchial epithelial cells, Proc Natl Acad Sci U S A 1986, 83:2438-2442

40. Olson EN, Sternberg E, Hu JS, Spizz G, Wilcox C: Regulation of myogenic differentiation by type beta transforming growth factor, J Cell Biol 1986, 103:1799-1805

41. Shen W, Li Y, Tang Y, Cummins J, Huard J: NS-398, a cyclooxygenase-2-specific inhibitor, delays skeletal muscle healing by decreasing regeneration and promoting fibrosis, Am J Pathol 2005, 167:1105-1117

42. Kozian DH, Ziche M, Augustin HG: The activin-binding protein follistatin regulates autocrine endothelial cell activity and induces angiogenesis, Lab Invest 1997, 76:267-276

43. Glienke J, Schmitt AO, Pilarsky C, Hinzmann B, Weiss B, Rosenthal A, Thierauch KH: Differential gene expression by endothelial cells in distinct angiogenic states, Eur J Biochem 2000, 267:2820-2830

44. McCarthy SA, Bicknell R: Inhibition of vascular endothelial cell growth by activin-A, J Biol Chem 1993, 268:23066-23071

45. Ahn A, Frishman WH, Gutwein A, Passeri J, Nelson M: Therapeutic angiogenesis: a new treatment approach for ischemic heart disease--part I, Cardiol Rev 2008, 16:163-171

46. Okada M, Payne TR, Zheng B, Oshima H, Momoi N, Tobita K, Keller BB, Phillippi JA, Peault B, Huard J: Myogenic endothelial cells purified from human skeletal muscle improve cardiac function after transplantation into infarcted myocardium, J Am Coll Cardiol 2008, 52:1869-1880

47. Payne TR, Oshima H, Okada M, Momoi N, Tobita K, Keller BB, Peng H, Huard J: A relationship between vascular endothelial growth factor, angiogenesis, and cardiac repair after muscle stem cell transplantation into ischemic hearts, J Am Coll Cardiol 2007, 50:1677-1684

48. Gros J, Manceau M, Thome V, Marcelle C: A common somitic origin for embryonic muscle progenitors and satellite cells, Nature 2005, 435:954-958

49. Kassar-Duchossoy L, Giacone E, Gayraud-Morel B, Jory A, Gomes D, Tajbakhsh S: Pax3/Pax7 mark a novel population of primitive myogenic cells during development, Genes Dev 2005, 19:1426-1431

50. Relaix F, Rocancourt D, Mansouri A, Buckingham M: A Pax3/Pax7-dependent population of skeletal muscle progenitor cells, Nature 2005, 435:948-953

51. Le Grand F, Rudnicki MA: Skeletal muscle satellite cells and adult myogenesis, Curr Opin Cell Biol 2007, 19:628-633

52. McCroskery S, Thomas M, Maxwell L, Sharma M, Kambadur R: Myostatin negatively regulates satellite cell activation and self-renewal, J Cell Biol 2003, 162:1135-1147

53. McFarlane C, Hennebry A, Thomas M, Plummer E, Ling N, Sharma M, Kambadur R: Myostatin signals through Pax7 to regulate satellite cell self-renewal, Exp Cell Res 2008, 314:317-329

54. Gharaibeh B, Lu A, Tebbets J, Zheng B, Feduska J, Crisan M, Peault B, Cummins J, Huard J: Isolation of a slowly adhering cell fraction containing stem cells from murine skeletal muscle by the preplate technique, Nat Protoc 2008, 3:1501-1509

55. Deasy BM, Lu A, Tebbets JC, Feduska JM, Schugar RC, Pollett JB, Sun B, Urish KL, Gharaibeh BM, Cao B, Rubin RT, Huard J: A role for cell sex in stem cell-mediated skeletal muscle regeneration: female cells have higher muscle regeneration efficiency, J Cell Biol 2007, 177:73-86

56. Kunkel LM, Beggs AH, Hoffman EP: Molecular genetics of Duchenne and Becker muscular dystrophy: emphasis on improved diagnosis, Clin Chem 1989, 35:B21-24

57. Bieber FR, Hoffman EP: Duchenne and Becker muscular dystrophies: genetics, prenatal diagnosis, and future prospects, Clin Perinatol 1990, 17:845-865

58. Benabdallah BF, Bouchentouf M, Rousseau J, Bigey P, Michaud A, Chapdelaine P, Scherman D, Tremblay JP: Inhibiting myostatin with follistatin improves the success of myoblast transplantation in dystrophic mice, Cell Transplant 2008, 17:337-350

59. Jankowski RJ, Deasy BM, Cao B, Gates C, Huard J: The role of CD34 expression and cellular fusion in the regeneration capacity of myogenic progenitor cells, J Cell Sci 2002, 115:4361-4374

Acknowledgments

The authors would like to thank Dr. Se-Jin Lee from Johns Hopkins University for kindly providing breeders of follistatin over-expressing mice, and Lynne Bauer for maintaining and breeding mdx/SCID mice, myostatin knockout mice and follistatin over-expressing mice. We would also like to thank James H. Cummins for his editorial assistance in preparing this manuscript.

Footnotes

The author, Jinhong Zhu, accomplished Ph.D in University of Pittsburgh in 2009, and currently is doing research as a postdoctoral fellow in the Genomic Medicine Institute, Cleveland Clinic, Cleveland, Ohio 44195, USA.

The abbreviation used are: TGF-β1, transforming growth factor –beta 1; ACVR2B, activin receptor II B; DMD, Duchene's Muscular Dystrophy; MyHC, myosin heavy chain; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; P/S, penicillin/streptomycin; H&E, Hematoxylin and Eosin; DAPI, dihydrochloride 4', 6-Diamidino-2-phenylindole dihydrochloride; FLST, follistatin; FLST/OE, follistatin over-expressing; MSTN, myostatin; MPCs, muscle progenitor cells; WT, wild type

Figure Legends

Fig.1. Injured follistatin over-expressing (FLST/OE) skeletal muscles showed accelerated regeneration as compared with wild type (WT) counterparts. A. HE staining of cross section of injured WT and FLST/OE skeletal muscle at 7, 14, 30 days and 1.5 years after laceration injury. Myofibers and nuclei were stained in red and black, respectively. Regenerating myofibers are characterized by centralized nuclei. Black bar represents for 100 μm. B. Distribution of diameters of regenerating myofibers in WT and FLST/OE skeletal muscle 7 (n = 3), 14 (n = 8), 30 (n = 8) days, and 1.5 years (n = 4) after post-injury. Gray bars represent myofibers from WT mice while black bars represent myofibers from FLST/OE mice. Moreover, the gray arrowheads indicate mean diameters of regenerating fibers in WT muscle, while black arrowheads indicate that in FLST/OE muscle. C. Quantitation of diameters of regenerating myofiber. The smallest diameters of Over 300 non-adjacent myofiber per muscle were measured by Northern Eclipse Software. The

increases in the mean diameters of regenerating fibers in FLST/OE muscle over that in WT muscle is increasing. ($P < 0.05$*, $P < 0.01$**)

Fig.2. Fibrosis in injured follistatin over-expressing (FLST/OE) muscle was reduced when compared to the injured wild type (WT) muscle. Masson's trichrome staining was performed on sections of injured FLST/OE and WT muscle (myofibers in red; fibrosis in blue). A. Representative images of injured FLST/OE and WT muscle at 14 ($n = 8$) and 30 ($n = 8$) days after injury. There is significantly less fibrosis observed in injured FLST/OE muscle than WT muscle. B. Injured FLST/OE muscles developed significantly less fibrosis than did injured WT muscles. ($P < 0.05$*, $P < 0.01$**)

Fig.3. Decreased myostatin (MSTN) expression and increased angiogenesis in injured follistatin over-expressing (FLST/OE) skeletal muscles. A. Immunohistochemistry was performed to detect MSTN (fluorescence red) and collagen type IV (fluorescence green) expression in injured wild type (WT) and FLST/OE muscle. Collagen type IV was used to highlight the basal lamina of myofibers including, necrotic, intact, and regenerating myofibers. MSTN-positive signals were seen with in some of regenerating myofibers with basal lamina (arrows) and some of myofibers without basal lamina (arrowheads). Injured FLST/OE muscle contained less MSTN staining than did injured WT muscle. B. When we measured the relative area of MSTN positive, we found that there were significantly more MSTN expression detected in injured WT muscle than in injured FLST/OE muscle. C. CD31, an endothelial maker, was stained to monitor capillaries in injured muscle. D. There were a significantly more CD31 positive capillaries in injured FLST/OE muscle than that in injured WT muscle ($n = 8$). ($P < 0.05$*, $P < 0.01$**)

Fig.4. Characterization of muscle progenitor cells (MPCs). Seven follistatin over-expressing (FLST/OE)- and 5 wild type (WT)-MPC populations were examined for Sca-1 expression, CD34 expression, and in vitro myogenic differentation. A. Histograms are showing wild variability in percentages of Sca-1+&CD34+ Sca-1+, and CD34+ cells among MPC populations. B. Quantitation revealed a significant increase in Sca-1+ fraction in FLST/OE-MPC population as compared with WT-MPC populations. C. Images on the left side are isotype control; images on the right side are representative images of flow plot showing that FLST/OE-MPC populations consist of large proportion of Sca-1+ cells than its WT counterpart (46.5% vs. 24%); D. Both FLST/OE- and WT-MPC populations underwent myogenic differentiation as labeled by myosin heavy chain (MyHC) (fluorescence red) and DAPI (blue).

Fig.5. Follistatin over-expressing-muscle progenitor cells (FLST/OE-MPCs) regenerated skeletal muscle more efficiently than wild type (WT)-MPCs, when transplanted into gastrocnemius muscles of mdx/SCID mice. A. Quantitation of engraftment in terms of number of dystrophin-positive fibers regenerated from FLST/OE- and WT-MPC populations. B. The overall mean dystrophin-positive myofibers was significantly larger for FLST/OE MPCs (592.79 ± 154.9; Mean ± SEM; n = 7 FLST/OE-MPC populations; 4 muscles per population) than for WT-MPCs (195.6 ± 65.375; Mean ± SEM; n = 5 WT-MPC populations; 4 muscles per population; P = 0.023**, t-test). C. Representative engraftments showed that transplanted MPCs regenerated dystrophin-postive myofibers (fluorescence red) within dystrophic muscle. FLST/OE-MPCs produced more dystrophin-positive myofibers than did WT-MPCs. (P < 0.05*, P < 0.01**)

Fig.6. Interactions between follistatin (FLST) and myostatin (MSTN), and FLST and activin A. A. As indicated by fusion index (FI, the ratio of nuclei in myotubes to total nuclei), MSTN significantly inhibited C2C12 myoblast differentiation, but, FLST counteracted MSTN and

attenuated it's inhibition on cell differentiation. B. Without intervention, C2C12 myoblasts underwent myogenic differentiation in low serum medium evidenced by myosin heavy chain (MyHC) and Dapi; Activin A significantly reduced muscle cell differentiation and the formation of myotubes in cell culture. C. FLST could neutralize inhibitory effect of activin A on myoblast differentiation shown by FLST induce-restoration of differentiation. ($n = 3$; $P < 0.05$*, $P < 0.01$**)

Fig.7. Follistatin (FLST) neutralized TGF-β1's activity on C2C12 myoblasts. A. Exemplary pictures of differentiation of C2C12 myoblasts treated by FLST alone, TGF-β1, and combinations of FLST and TGF-β1. Myotubes were visualized by myosin heavy chain (MyHC) (fluorescence red), and nuclei were blue (Dapi). B. Our quantitative results showed that TGF-β1 significantly inhibited myogenic differentiation of C2C12 myoblasts. FLST was able to reverse TGF-β1-inhibited myogenic differentiation ($n = 3$). C. Western blot results showed that FLST decreased TGF-β1 expression in C2C12 with or without the presence of exogenous TGF-β1. D. FLST also reduced expression and phosphorylation of SMAD2. E. FLST stimulated expressions of myogenic regulatory factors, MyoD, Myf5, and myogenin by myoblasts; FLST reduced MSTN expression by myoblasts (arrowheads). ($P < 0.05$*, $P < 0.01$**)

AAV-Mediated Myostatin Propeptide Gene Transfer Improved the Healing of Laceration Injured Skeletal Muscle and Muscle Progenitor Cell Transplantation Efficiency

Jinhong Zhu, M.D., Ph.D.,*[£] Jianqun Ma, M.D., Ph.D.,*[†‡§] Chunping Qiao, M.D.,[ξ] Jianbin Li, M.D.,[ξ] Andres J. Quintero, M.D.,*[†] Yuri Chun, M.D., *[†] Aiping Lu, MD., *[†] Burhan Gharaibeh, Ph.D.,*[†] Yong Li, M.D., Ph.D.,*[†‡] Xiao Xiao, M.D., Ph.D.,[ξ] & Johnny Huard, Ph.D*[†£].

Stem Cell Research Center, Children's Hospital of Pittsburgh, PA,*

Department of Orthopaedic Surgery, University of Pittsburgh, PA, [†]

Department of Bioengineering, University of Pittsburgh, PA, [£]

Lab of Molecular Pathology, Department of Pathology, University of Pittsburgh, PA [‡]

Division of Molecular Pharmaceutics, University of North Carolina School of Pharmacy, Chapel Hill, NC,[ξ]

Division of Thoracic surgery, the Affiliated Tumor Hospital of Harbin Medical University, Harbin, China[§]

Running head: Myostatin Propeptide Improved Muscle Healing

Number of text page: 27 **Number of figures:** 7

Corresponding Author:
Dr. Johnny Huard
Henry J. Mankin Professor and Vice Chair for Research
Department of Orthopaedic Surgery,
Professor, Departments of Molecular Genetics and Biochemistry and Bioengineering
Director, Stem Cell Research Center
Children's Hospital of Pittsburgh
4100 Rangos Research Center
3640 Fifth Avenue, Pittsburgh, PA 15213-2582
email: jhuard@pitt.edu; Ph: 412-648-4066; Fax: 412-692-2782

Abstract

The development of approaches to improve muscle healing after injuries has focused largely on inhibiting fibrosis and promoting myogenesis. Since the inhibition of myostatin (MSTN), a negative regulator of skeletal muscle, causes a remarkable increase in skeletal muscle mass, we posited that MSTN blockade—with MSTN propeptide (MPRO) could improve muscle healing after injury. Our results demonstrated that the injection of an adeno-associated viral (AAV) MPRO vector into normal skeletal muscle, 4 weeks prior to creating a laceration injury in the muscle, led to an improvement of muscle healing when analyzed 4 weeks post-laceration and compared to the control groups. This effect was also observed in long term experimental animals which were sacrificed 1 year post-laceration. Next we explored the potential mechanisms by which MSTN blockade improved skeletal muscle healing. I*n vitro* we observed an enhancement of myoblasts' ability to differentiate into myotubes after AAV-2-MPRO gene transfer. We also demonstrated that muscle progenitor cells (MPCs) isolated from MSTN -/- mice regenerated significantly more myofibers than MPCs isolated from wild type (WT) mice when injected into the dystrophic skeletal muscle of mdx mice. Our results also suggest that MPROs beneficial effects are related to an increase in muscle regeneration, a reduction in fibrosis deposition and an increase in capillary in-growth into the injury site. These results suggest that MSTN blockade has a beneficial effect on muscle healing through an enhancement of the myogenic potential of MPCs as well as through the amelioration of the local environment within the injured skeletal muscle.

Introduction

Myostatin (MSTN), a member of the transforming growth factor-β (TGF-β) superfamily, is the key negative regulator of fetal and adult skeletal muscle growth[1], and is highly conserved across different species ranging from zebra fish to human [1-9]. Deficiency of MSTN function causes a remarkable increase in skeletal muscle mass from hypertrophy [2, 7, 9] to both hypertrophy and hyperplasia [1, 3-5, 8]. Therefore, various therapeutic strategies for treating muscular dystrophies and muscle wasting disorders have been developed to target MSTN. The *mdx* mouse, an animal model of Duchenne muscular dystrophy (DMD), displays a cycling of muscle degeneration and regeneration due to the absence of dystrophin expression and later in life the skeletal muscle of these mice show a remarkable accumulation of fibrotic and fatty tissues. Research has shown that *mdx* mice that have their MSTN gene knocked out have greater muscle mass, are physically stronger, have less fibrosis, and possess a superior capacity for skeletal muscle regeneration [10]. Similar functional and histopathological improvements have been observed in *mdx* mice treated with an antibody that blocks MSTN and using recombinant myostain propeptide (MPRO) [11-12]. Subsequently, a variety of inhibitors of MSTN have been identified, including as MSTN blocking antibody, MSTN MPRO [8-9, 13-14], follistatin (FLST) [8, 13], follistatin-related genes [14], growth and differentiation factor-associated serum protein-1 [15], and soluble activin type IIB receptor (ACVR2B) [16].

Several groups, including ours, have found that MSTN positively regulates the formation of skeletal muscle fibrosis, much as is the case with TGF-β1 [10, 17-19]. Consistent with this are the findings that recombinant MSTN protein stimulates fibroblasts to proliferate and synthesize TGF- β1, and fibrotic proteins such as fibronectin, and collagen types I and III [18]. *In vivo*, injured MSTN knockout (MSTN-/-) mouse skeletal

muscles not only exhibit improved muscle regeneration, but also develop significantly less fibrosis after injury than their wide-type (WT) controls; this is in part because MSTN carries out a signal transduction similar to the pro-fibrotic signaling of TGF-β1 [20]. In fact, it appears that TGF-β1 and MSTN act synergistically [18, 21] to induce fibrosis in injured muscle [18]. MSTN is therefore an ideal therapeutic target for enhancing regeneration and reducing the formation of fibrosis in injured and diseased skeletal muscle.

Analogous to TGF-β1, MSTN is synthesized as a precursor protein consisting of a signal sequence, an N-terminal propeptide domain, and a C-terminal domain. After two proteolytic cleavages remove the signal sequences and propeptides, two C-terminal domains form a disulfide-linked dimer, which is the active form of MSTN [1, 22]; however, two released propeptide molecules are able to non-covalently bind to one MSTN dimer and inhibit the biological activity of MSTN [8, 13]. Activation of MSTN requires the release of its mature dimer from the latent complex [8]. Different research groups have shown that over-expression of MPRO in transgenic animals increases muscle regeneration [7-9]. Although the amount of increase in muscle mass may vary, it appears to be proportional to levels of transgenic gene expression [8-9]. It was also found that recombinant MPRO binds to MSTN and antagonizes its biological activity [23-24], so that the local and systemic administration of MPRO can both successfully block MSTN activity and attenuate the severity of skeletal muscle dystrophy [12, 25-26].

In this current study, we investigate the mechanism by which MSTN blockade with MPRO improves muscle regeneration and repair after injury. We used an adeno-associated virus serotype 2 (AAV2) to deliver MPRO to the laceration injured skeletal muscle of mice and examined the effect that MPRO had on muscle regeneration and

repair and the potential influence that the MPRO had on muscle progenitor cells and the influence it had on the local environment.

Materials and methods

Construction of an AAV vector encoding for MPRO gene

The AAV2-MPRO particle, encoding a MSTN propeptide (MPRO) sequence under the control of the cytomegalovirus (CMV) promoter was generated following a co-transfection method described previously [27]. We used PCR to amplify MPRO cDNA, and than cloned it into a CMV promoter-driven AAV vector plasmid [26]. A pair of primers was designed to amplify the gene segment spanning the 5' portion of the MPRO cDNA to detect AAV2-MPRO transcription. The sequences of primers for CMV-MPRO are: forward: AAGCTGCAGAAGTTGGTCG, and reverse: AGTGGAGGCGCTCT-TGGC [26]. In vitro expression of AAV-MPRO in C2C12 myoblasts was examined by RT-PCR.

Transduction of AAV2-MPRO in C2C12 myoblasts in vitro

C2C12 myoblasts were plated on collagen-coated 12-well plates, grown overnight, and then infected with the AAV2-MPRO vector at a dose of 10^6 AAV2-MPRO viral genomes (v.g.)/cell. The cells were then cultured for 4 additional days in low serum containing medium. Following fixation of cells, myosin heavy chain (MyHC) immunostaining was performed to monitor myotube formation, and the fusion index was calculated by determining the ratio of nuclei within fused myotubes ($\geq$ 2 nuclei) to the total number of nuclei.

Western blot analyses

Total protein was extracted from cells with Laemmli sample buffer (Bio-Rad, Hercules, CA) supplemented with β-mercaptoethanol. Equal amounts of the protein samples were loaded and separated on 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gel. Separated protein bands were transferred to nitrocellulose membranes (Bio-Rad) by electroblotting, which was followed by a 2h blocking in 0.05% Tween-20 phophate buffered saline containing 5% nonfat milk at room temperature (RT). Primary antibodies including mouse anti-MyoD (554130, 1:250; BD Pharmingen; San Diego, CA), anti-myogenin (556358, 1:250; BD Pharmingen; San Diego, CA), and goat anti-Myf5 (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were applied to the membranes and incubated at RT overnight -- mouse anti-ß-actin was used as a loading control (1:8000, Sigma; St. Louis, MO). The following day, after sufficient wash <<With what, PBS??>>, the membranes were incubated with horse-radish peroxidase (HRP)-conjugated secondary antibodies (Pierce, Rockford, IL) diluted to 1:5000 and applied. HRP activity was determined by its reaction with SuperSignal West Pico chemiluminescent substrate (Pierce), and finally positive bands were visualized on X-ray film in a dark room. All results were analyzed with Northern Eclipse software (Empix Imaging; Cheektowaga, NY)

Intramuscular injection of AAV2-MPRO/GFP into wide type (WT) mice

Ten male C57BL/6 WT mice (8 weeks old) were used for these experiments. Fifty μl of AAV2-MPRO ($2.5x10^{12}$ v.g./ml) was injected into both Gastrocnemius Muscles (GMs) of 10 mice; the same amount of phosphate buffered saline (PBS) was injected into the GMs of 5 mice as controls. One month after AAV2 vector transduction, both GMs of each mouse were subjected to a laceration injury as described previously[18]. Mice were sacrificed at 4 weeks after laceration and the GMs were harvested and cryogenically

snap frozen in Methylbutane cooled in liquid nitrogen <<is this correct??>>. CD31, an endothelial marker, staining was used to assess whether MPRO over-expression would increase vascularity in the injured muscle when compared to the WT control.

In a long-term study, GMs of 2 C57BL/6 WT mice (8 weeks old, n = 4) were injected with 50 μl of AAV2-MPRO ($2.5x10^{12}$ v.g./ml), whereas, the same dose of AAV2-GFP was injected into GMs of 2 littermates (n = 4) as controls, which was followed by bilateral laceration of GMs at 4 weeks post viral transduction. Mice were then sacrificed at one year post-injury. Muscle regeneration and fibrosis were measured for both sets of animals according to a published protocol [18].

Isolation of Muscle progenitor cells from WT and MSTN-/- mice and cell transplantation << I don't think we should remove this section>>

Muscle progenitor cells (MPCs) were isolated based on their properties of slow-adherence to collagen-coated flask using a published modified preplate technique [28-29]**.** We isolated MPCs from 5 male neonatal C57BL/6J WT mice as well as 5 MPC populations isolated from 5 male neonatal MSTN-/- mice. Briefly, newborn mice were sacrificed, and both upper and lower limbs were harvested. After removal of skin, bone, cartilage, the remaining muscles were subjected to sequential enzymatic digestions including: collagenase type XI, dispase, and trypsin. The resulting muscle cell extracts were then seeded on collagen-coated flasks. Different cell populations were obtained by serially replating the extracts at different time intervals (2h (PP1), 24h (PP2), 24h (PP3), 24h (PP4), 24h (PP5), and 72h (PP6)). PP1 cells are mostly fibroblasts. The earlier preplate populations (PP2-5) are mainly composed of myoblasts and satellite cells, whereas the late preplate populations (PP6) attached to flask within the last 72 h enriches MPCs [28]. Late preplate cells were collected as MPCs, grown at a low population density in proliferation medium (Dulbecco's modified Eagle's medium

supplemented with 1% penicillin-streptomycin, 10% fetal bovine serum, 10% horse serum, and 0.5% chicken embryo extract), and used for *in vivo* cell transplantation.

We expanded the WT and MSTN-/- MPC populations in vitro in PM. When the cells reached a given density, we trypsinized the cells, washed them with PBS, and then counted them with a hemocytometer. The MPC populations were then injected intramuscularly into GMs of female *mdx*/SCID mice – a dystrophic, immuno-incompetent mouse model that was generated by crossing *mdx* (C57BL/10ScSn-*Dmd*^{mdx}, Jackson laboratory) and SCID mice (C57BL/6J-*prkdc*^{scid}/SzJ mice, Jackson laboratory) at the animal facility of our institution. Each MPC population was transplanted into skeletal muscle of 4 legs of *mdx*/SCID. Approximately 300,000 MPCs were transplanted into each GM of female mice. At two weeks after cell transplantation, the mice were sacrificed, and GMs were harvested, snap frozen and cryosectioned. Dystrophin immunostaining was performed and the number of dystrophin-positive fibers was used to evaluate muscle regeneration capacity of transplanted donor cells.

Intramuscular injection of AAV2-MPRO/GFP into mdx/SCID mice prior to WT MPC transplantation

To evaluate whether blocking MSTN signaling in host dystrophic muscle using MPRO can improve the regeneration capacity of WT-MPCs, AAV2-MPRO ($2.5x10^{12}$ v.g./ml), in 50 µl of PBS, was injected into both GMs of 3 *mdx*/SCID mice (4 weeks of age, n = 6) 4 weeks prior to cell transplantation; while 3 littermates received the same dose of AAV2-GFP (n = 6) as controls. Muscle progenitor cells (MPCs), isolated from the skeletal muscle of C57BL/6 WT mice, were transplanted into both AAV-MPRO and AAV-GFP-transduced GMs (300,000 cells per GM). Before cell transplantation, MPCs were trypsinized and characterized by flow cytometry for CD34 and Sca-1 expression. To

label these cells, MPCs were blocked with 10% mouse serum, and then incubated with rat anti-mouse Sca-1 PE and biotinylated CD34 monoclonal antibodies (ABCAM; Cambridge, MA) followed by an incubation with streptavidin (SA)-APC (BD Pharmingen; San Diego, CA). Control cells were treated with corresponding isotype control antibodies. Before analysis, 7-amino-actinomycin D was added to exclude the nonviable cells. Flow cytometry (FACStar Plus or FACSAria; Becton Dickinson; Franklin Lakes, NJ) was used to determine the percentages of CD34 and Sca-1 positive cells. Moreover, the MPCs' desmin expression and myogenic differentiation capacity were evaluated by immunocytochemistry. For examination of desmin, MPCs were plated on 12 well plates at a density of 10,000 cells per well and grown in PM overnight prior to staining. Moreover, for myogenic differentiation analysis, MPCs were plated on 12 well plates (20,000 cells per well) with DMEM supplemented with 2% FBS (low serum containing medium stimulates myotube differentiation) and cultured for 4 days. Prior to staining, cells were fixed with pre-cooled methanol for 2 min, blocked with 10% horse serum for 30 min, and then incubated with a mouse anti-desmin antibody, or a mouse anti-myosin heavy chain (MyHC) antibody (1:250,M-4276, Sigma; St. Louis, MO) for 2 hours. The primary antibodies were detected with a secondary anti-mouse IgG antibody conjugated with Cy3 (1:300, C2181, Sigma; St. Louis, MO) for 1h. The nuclei were stained with DAPI and the percentage of desmin positive cells was determined. The myogenic differentiation capacity of the cells was determined using the fusion index as described previously.

Two weeks post-cell transplantation, mice were sacrificed and the GMs were harvested for immunohistochemistry and histological examination. MPC transplantation efficiency was measured by analyzing the number of dystropin-positive myofibers

present in the host (dystrophin deficient) muscle. Fibrosis was determined by Masson's trichrome staining; muscle regeneration was evaluated after H&E staining; in a separate set of experiments. *Mdx*/SCID mice were sacrificed 3 months post AAV-MPRO/GFP injection.

Immunohistochemistry

To stain for CD31 or dystrophin, tissue sections were fixed in 4% formalin for 5 minutes followed by two 10-minute washes with PBS. The sections were then blocked with 10% HS for 1 hour. The rat CD31 primary antibody (BD Pharmingen; San Diego, CA) was diluted 1:150 in 2% HS, or a rabbit dystrophin primary antibody (1:400, Abcam; Cambridge, MA) and incubated with sections for 1h at room temperature. The sections were washed three times with PBS and then incubated with the corresponding secondary antibody, rabbit anti-rat IgG conjugated with Alexafluor 555 (red, Invitrogen; Carlsbad, CA) or donkey anti-rabbit IgG conjugated with Alexafluor 594 (red, Invitrogen; Carlsbad, CA) for 30 minutes. DAPI was used to stain the nuclei.

To perform MPRO and collagen IV double-immunostaining, tissue sections were fixed and blocked following the standard protocol described above. Sections were then incubated overnight at 4^{o}C with a goat MPRO antibody (2.5 ug/ml, RnD system) that was diluted in 5% donkey serum. The following day, these sections were washed with PBS and incubated with the secondary antibodies, rabbit anti-Goat IgG conjugated with Alexafluor 594 (red, Invitrogen; Carlsbad, CA). The MPRO stained sections were then blocked again prior to the use of collagen IV primary antibody. Rabbit anti-Collagen IV IgG (1:250, Biodesign International, Saco, ME) in 5% serum was applied on the sections for 1h at RT followed by <<how many??>>PBS washes and incubation with donkey anti-rabbit IgG conjugated with Alexafluor 488 (green, Invitrogen; Carlsbad, CA).

Statistics

All of the results from this study are expressed as the mean ± standard deviation (SD) or mean ± error of the mean (SEM). The differences between means were considered statistically significant if $P < 0.05$. The Student's t-test was used to compare the difference between two groups. Error bars on the graphs represent the SD or SEM. (*) represents $P < 0.05$, and (**) represents $P < 0.01$.

Results

Direct in vivo injection of AAV2-MPRO inhibits MSTN activity in skeletal muscle

In order to test if we could improve the healing process of acutely injured skeletal muscle by blocking MSTN, we injected the AAV-MPRO vector directly into the GMs of adult C57BL6/J mice -- as a control, we injected the same amount of PBS into age matched littermates. Four weeks after AAV2 vector delivery both GMs were lacerated and the mice were sacrificed 4 weeks post-injury. Masson's Trichrome staining showed extensive fibrosis infiltration in the injured WT skeletal muscle, while injured AAV-MPRO transduced muscle formed significantly less fibrotic tissue (Figure 1, A, B, (7.21 ±1.06 vs. 2.64 ± 1.04.; mean ± SD, $^{**}P < 0.01$)). Figure 1, C demonstrates the stable expression of MPRO in the AAV transduced muscles. The weight of AAV2-MPRO transduced-GMs was also significantly greater than that of the untransduced WT counterparts (Figure 1, D). Moreover, we observed larger regenerating myofibers in the AAV2-MPRO transduced injured muscle than in the injured WT muscle (Figure, 1, E). The mean diameter of regenerating myofibers in the GMs over-expressing MPRO was significantly increased by 26.7% more than what was observed in the non-transduced GMs (36.79 ± 3.79 vs. 29.03 ± 2.18; mean ± SD, $^{**}P < 0.01$) (Figure, 1, F). Correspondingly, the distribution of the diameters of regenerating myofibers revealed that 78% of the regenerating myofibers in the control muscles had a diameter smaller than 35 µm when

compared to 46.28 % of that in AAV2-MPRO transduced muscles; by contrast, 53.72% of the regenerating myofibers in injured AAV-MPRO transduced muscle had a diameter in the range of 35 to 70 µm (Figure 1, G).

Long term beneficial effects of MPRO on muscle healing

In a separate study, we injected either the AAV-GFP or the AAV-MPRO into the GMs of different littermates 4 weeks prior to injuring the muscles. The animal were sacrificed one year after lacerating the muscles and our results demonstrated that 1 year after gene transfer, GFP and MPRO were constitutively expressed in the myofibers of the transduced muscles. We observed the GFP-positive fibers in the AAV-GFP treated muscle, but not in the AAV-MPRO-treated muscle (Figure, 2, A). Collagen type IV staining outlines the myofibers; MPRO-positive myofibers (arrowheads) indicate a constitutive expression of MPRO in skeletal muscles transduced with the AAV-MPRO, but not in the AAV-GFP control muscle (Figure, 2, A). Many regenerating myofibers remained centronucleated in muscles over-expressing GFP or MPRO 1 year post-injury (Figure, 2, B). We also observed more regenerating myofibers that were also significantly larger in the AAV2-MPRO transduced injured muscle than in the controls (Figure, 2, B). For instance, 30% of the regenerating myofibers in the control muscle were larger than 50 µm when compared to 61 % in the AAV2-MPRO transduced muscles (Figure, 2, C). Finally, the blockade of the MSTN with MPRO also has a long-term effect with a significant increase in the weight of GMs 1 year after gene transfer (Figure, 2, D).

AAV2-MPRO inhibits MSTN activity in vitro

In this section of the study, we used an adeno-associated viral vector serotype 2 (AAV-2) to deliver the MPRO gene to C2C12 myoblasts *in vitro*. RT-PCR results show that the C2C12 cells successfully expressed the MPRO gene (Figure, 3, A). MPRO

effectively neutralized MSTN and stimulated C2C12 myoblasts to differentiate and readily fuse into larger myotubes containing numerous myonuclei and had a significant increase in their cell fusion index when compared to the non transduced C2C12 myoblasts (Figure, 3, B, C). Moreover, an over-expression of MPRO stimulated the expression of Myf5 and myogenin in C2C12 myoblasts in differentiation medium, without affecting MyoD expression (Figure, 3, D).

In the next set of experiments we wanted to examine if there was a similar increase in the regeneration capacity of chronically damaged muscle, dystrophic muscle, when we blocked MSTN with AAV-MPRO. We compared the differences in dystrophic pathology between the GMs of *mdx*/SCID mice transduced with either AAV-GFP or MPRO. Cryosectioned muscle samples harvested at 6 weeks after virus transduction were stained with H&E and Masson's trichrome. Histology showed a decreased dystrophic phenotype after the injection of AAV-MPRO. The insets in figure 7A (20x) show whole sections of GMs; the arrows in the insets highlight that there were more degenerative foci resulting from pathological changes observed in the AAV-GFP-transduced control dystrophic muscle than the AAV-MPRO-treated dystrophic muscle, which were poorly stained by eosin (Figure, 3, A). The enlarged images (40x) revealed that the areas which demonstrated the pathological phenotype in the control treated muscle consisted of numerous mononucleated cells, connective tissue, or small regenerating myofibers. The necrotic myofibers and inflammatory cell infiltration can be readily appreciated in a further enlarged image (100x) of the control muscles (arrows and arrowhead), whereas the AAV-MPRO-treated muscle exhibited less damage and more regenerating fibers (arrows and arrowhead, Figure, 3, A). Additionally, Masson's trichrome stain reveals that the degenerative areas were associated with extensive

~~fibrotic tissue (blue); low magnification images (2x) further revealed that the control dystrophic muscles generally contained more fibrotic foci (white arrows) than the AAV-MPRO-treated dystrophic muscles. There is more fibrosis in the control muscles -- averaging 7.08% (and varying from 5.32 to 9.02%) by area of GM cross-section affected -- as compared to that noted in the MPRO treated muscle, which averaged 4.09% (varying from 2.73 to 6.07%). There was also a significant decrease in fibrous scar tissue in the AAV2-MPRO treated dystrophic muscles as compared to the controls (Figure, 3, B). We also observed hypertrophy in AAV2-MPRO-treated dystrophic muscle, with the mean diameter of these muscles being significantly larger than that of the AAV-GFP-transduced control counterparts (Figure, 3, C). The AAV2-MPRO transduction-induced muscle hypertrophy consequently leads to a significant increase in muscle weight when compared to controls (Figure, 3, D).~~ The histo-morphological improvements in dystrophic muscle treated with AAV-MPRO is related to a decrease in the amount of cellular infiltration, necrosis, and fibrosis, and an increase in the diameter of muscle fibers that contribute to an increase in muscle weight.<<Perhaps include a paragraph in discussion as data not included>>

High muscle regenerative potential was exhibited by MPCs isolated from MSTN-/- mice

In this study we further demonstrated that MSTN has a negative regulatory effect on the cells participating in the muscle regeneration process by isolating MPCs from MSTN-/- mice and comparing them to WT MPCs. We isolated MPCs from the skeletal muscle of the MSTN-/- mice compared them to WT MPCs when they were injected into the skeletal muscle of dystrophic mdx/SCID mice. We isolated five WT MPC populations and five MSTN-/- MPC populations from five WT and MSTN-/- mice, respectively. Each

cell population was injected into the skeletal muscle of *mdx*/SCID mice. Transplantation of MSTN-/- MPCs into the dystrophic muscle regenerated more dystrophin-positive myofibers when compared to WT MPCs. Although we observed variability in the number of regenerated dystrophin expressing muscle fibers among the different cell populations, three out of five MSNT-/- populations generated more than 400 myofibers, and two MSTN-/- MPC populations regenerated around 800 regenerated myofibers, while only one population of WT MPCs regenerated 400 dystrophin positive muscle fibers. As a result, the average number of regenerating myofibers in MSTN-/- MPC populations was superior to the WT cells (195.6 ± 65.357 vs. 518.08 ± 117.64; mean ± SEM, $P < 0.05$) (data not shown). <<Should we have a Figure for this section??>>

AAV-MPRO improves cell transplantation and reduces the severity of muscular dystrophy in mdx/SCID mice

In the next set of experiments we examined whether blocking MSTN in dystrophic host muscle with AAV-MPRO could improve the regeneration index of WT MPCs transplanted into the skeletal muscle of mdx/SCID mice. We transplanted MPCs isolated from WT mice into dystrophic muscles that were pre-treated with either AAV-GFP or AAV-MPRO 4 weeks prior to cell transplantation. *In vitro* characterization revealed that 70% of the MPCs expressed desmin (Figure, 4, A), and readily differentiated into multi-nucleated myotubes, when cultivated in low serum containing medium (Figure, 4, B). A small portion of this MPC population was CD34 and Sca-1 double positive (Figure, 4, C, E). *In vivo* characterization showed that these MPCs were able to generate dystrophin positive myotubes when injected into dystrophic muscle of *mdx*/SCID mice (Figure, 4, D). Two weeks after cell transplantation, we examined whether blocking MSTN signaling by MPRO in host muscle increased the efficiency of

muscle cell transplantation in dystrophic muscles. Figure 6F shows the dystrophin-positive engraftments in the muscles of mdx/SCID mice 2 weeks after cell transplantation. We found that the number of dystrophin expressing muscle fibers, following the injection of WT MPCs into the AAV-MPRO-transduced dystrophic muscles, was significantly higher than the injection of WT MPCs into untransduced control muscles (Figure, 4, F).

<<This was the figure JH considered using for FLST paper…I think maybe we should keep in this paper and simply reference this in the FSLT paper…I think it fits fairly well here in telling the mechanistic story of MSTN blockade…if we keep Figs will be 5 >>

MSTN-/- muscles exhibit more CD31-positive capillary-like structures than WT muscle after injury

The sequence of events following skeletal muscle injury is well characterized. Following injury, ruptured myofibers shrink to cause the formation of a gap (central zone, CZ) between the end of myofibers, which is soon filled with a hematoma [31, 32]. Damaged myofibers, 1-2 mm from the ends of the ruptured myofibers, undergo necrosis, after which they regenerate within the regeneration zone (RZ). Undamaged parts of the muscle are called the survival zones (SZ). As regenerating myofibers extend themselves into the CZ , excess extracellular matrix is deposited in the CZ to form fibrotic connective tissue which hinders muscle regeneration [31, 32].

We previously reported that the injured skeletal muscle of MSTN-/- mice heals better than their age matched WT counterparts [18]. To investigate the underlying mechanism for this difference, we compared the neovascularization of injured WT and MSTN-/- muscles. CD31 (endothelial cell maker, red) and myosin heavy chain (MyHC, green) staining were carried out to determine the extent of angiogenesis in the injured skeletal muscle.

On post-laceration day #3, we observed numerous mononuclear cells and myofiber debris (black arrows in HE staining; white arrows in immunostaining) in the injured WT and MSTN-/- muscles (Figure, 5, B-a,a'). On day 5, the CD-31 positive capillaries could not be detected in the CZ of WT and MSTN-/- muscles, as delineated by white arrowheads (Figure, 5, B - b, b'). At 7 days post-injury, there was neovascularization present within the Central Zone of Injury (CZI) of MSTN-/- muscle, as noted by the ingrowth of capillary-like structures from the Regeneration Zone of Injury (RZI) and newly regenerated myofibers (black arrows in HE staining; white arrows in immunostaining) were penetrating into the CZI (Figure, 5, B-c'). By contrast, capillary-like structures were still absent in the CZI of WT muscle (arrowheads, Figure, 5, B-c). Corresponding H&E stain showed the histology of injured muscles at different time points after injury.

Moreover, we observed an irregular scattering of dilated capillary-like structures by the 4th week in WT muscles but more evenly distributed capillary-like structures were found in MSTN-/- muscle. A quantitative analysis demonstrated that injured MSTN-/- muscle showed a significantly higher capillary density than did the WT control (Figure 5, C). These results indicate that, in the absence of MSTN, the process of neovascularization after skeletal muscle injury, occurs more rapidly in MSTN-/- muscle than in the WT counterpart muscle.

MPRO blockade of MSTN produces results similar to those seen in MSTN-/- mice

To further investigate if blocking myostain with MPRO had a similar effect on neovascularizton we investigated whether laceration injured MPRO transduced muscle exhibited an increase in vascularity after injury, as was observed in the MSTN-/- mice. Figure 6 shows the H & E staining of MPRO transduced muscle as well as the control muscle after injury (Figure, 6, A). The double MyHC-CD31 staining illustrates

regenerating myofibers interspersed with CD31-positive capillaries at the injury site, which is shown in Figure, 6, B. The MPRO-over-expressing injured muscles displayed a higher density of capillary-like structures (CD31 positive) compared to the untransduced control counterpart muscles (Figure, 6, C) which was found to be statistically significant at 4 weeks post-injury (Figure, 6, D).

Discussion

Skeletal muscle injuries often occur in sports, constituting 35-55% of all sports-related injuries. The complete recovery of skeletal muscle after injury continues to pose a constant challenge for orthopaedic and rehabilitation physicians alike. After injury, skeletal muscle is able to undergo regeneration from myogenic progenitor cells such as satellite cells, and stem cells; however, severely injured muscle typically does not heal correctly and results in incomplete skeletal muscle regeneration. The ensuing formation of fibrosis hinders effective muscle regeneration where dense fibrosis impedes the growth of newly regenerating myofibers by preventing the ends of regenerating myofibers to extend into the CZ site and also preventing the diffusion of nutrients to the myofibers [30-31]. Thus, an approach to optimize the treatment of injured muscle should not only be able to enhance muscle regeneration, but also reduce the formation of fibrosis. We have used numerous anti-fibrotic agents, such as suramin, interferon-γ, decorin, relaxin, and losartan which block the actions of TGF-β1 and thereby blocks the formation of fibrosis and improves muscle healing after sports related muscle injuries including strain, contusion and laceration injuries [32-39].

The discovery of MSTN, a negative muscle regulator, represents a significant breakthrough for the potential development of therapeutic approaches for the treatment of myopathies. In addition to mounting evidence showing that inhibiting MSTN enhances muscle growth and regeneration undergoing pathological degeneration associated with muscle injury and muscular dystrophies [10-12, 17-18], MSTN also regulates skeletal muscle fibrosis deposition [17-19]. Without MSTN, as seen with MSTN -/- mice, skeletal muscle fibrosis is significantly reduced after injury than is observed in WT skeletal muscle [17-18]. While fibroblasts are the main source of extracellular matrix (ECM) deposition during the

formation of fibrosis, MSTN directly stimulates the proliferation of muscle fibroblasts and their *in vitro* and *in vivo* production of ECM [17-19]; therefore, MSTN is an excellent therapeutic target for treating injured and diseased muscles. Because the *in vivo* cytokine networks are very complex the underlying mechanisms by which MSTN inhibition attenuates muscle fibrosis requires further investigation. We have previously reported that MSTN and TGF-β1 synergistically induce fibrosis after skeletal muscle injury and the reduced fibrosis in injured MSTN-/- muscle may result, at least in part, from the absence of MSTN [18] or through another mechanism that involves MSTN.

The formation of new blood vessels is critical for wound healing [40-41]. Local tissue vascularity promotes skeletal and cardiac muscle regeneration and helps to reduce the formation of fibrosis following injury [42-46],, indeed, we noted an earlier onset of neovascularization from the time of injury in the MSTN-/- muscle compared to wild type muscle. At 4 weeks after injury, injured MSTN-/- muscle contained significantly more CD31-positive capillary-like structures than controls. The increased vascularity appears to partially account for the improved muscle healing observed in injured MSTN-/- mice. The use of transgenic animals has become a powerful and exciting research tool to study protein function and molecular mechanisms through the use of "loss or gain of function" experiments; however, it is worth mentioning that the irreversible genetic manipulations can result in compensatory up-regulations of other proteins, developmental defects, and other phenotypic anomalies. Changes take place during the prenatal life which may impose undesired effects on adult animals [47] ;therefore, an alternative experimental approach is the use of a virus as a gene delivery vehicle carrying the desired cDNA to specific tissues or organs. The adeno-associated virus (AAV) is garnering significant interest as a vector for human gene therapy, due to its

reduced immunogenicity and its ability to efficiently infect differentiated skeletal muscle fibers and stably express the transgene long term[48-49]. In this study, we injected an AAV2-mediated MPRO cDNA into the GMs of normal WT mice 4 weeks prior to injuring the muscles to establish a postnatal, muscle specific MSTN propeptide over-expression model.

Our results suggest that the AAV-MPRO gene could be stably expressed *in vivo* and improve the healing of injured muscle over an extended period of time. We found that MPRO gene transfer improved skeletal muscle healing by stimulating muscle regeneration and inhibiting fibrosis 4 weeks after injury, which coincided with the higher density of CD-31 positive capillary-like structures observed in MPRO transduced muscle verses untransduced controls. This further suggests that there is a negative correlation between MSTN and vascularity in injured skeletal muscle which is consistent with what we observed in the injured MSTN-/- muscle; however, the mechanisms for this correlation requires further investigation. The long term studies we performed also showed that there is abundant expression of the GFP and MPRO in laceration injured skeletal muscles 1 year post-injury. By that time the area of fibrosis had nearly disappeared in all injured muscles, while the hypertrophy of regenerating myofibers in AAV-MPRO transduced muscles was still very evident, indicating a continuous beneficial effect imparted by the AAV-MPRO on skeletal muscle.

The potential beneficial effect of blocking MSTN in injured and diseased skeletal muscle may be related, at least in part, to the function of resident muscle progenitor cells (MPCs). In fact a recent study performed by Tremblay *et al,* demonstrated that myoblasts isolated from transgenic mice that carry a dominant negative form of the myostatin receptor (dnActRIIB), gave rise to 75% more dystropin-positive fibers in

recipient dystrophic muscle than did myoblasts isolated from WT mice[50]. We have therefore tested whether blocking MSTN signaling influences the regeneration index of MPCs after their implantation into the dystrophic skeletal muscle of mdx/SCID mice.

We have previously reported that muscle regeneration in *mdx*/SCID mice is superior when their GMs are implanted with MPCs isolated from MSTN-/- mice as opposed to WT MPCs [18]. In the present study, we isolated more MSTN-/- MPC populations from different MSTN-/- mice and more extensively compared their regeneration potential after cell transplantation and compared them to the WT MPC populations. In this comparison study, we first found that there existed a broad heterogeneity within both WT MPCs and MSTN-/- MPC populations in terms of their capacity to regenerate muscle. This heterogeneity likely resulted from variations among the mice from which the cells were isolated, as well as the existence of variations among the host *mdx*/SCID mice; however, even with this heterogeneity we found that the MSTN-/- MPCs could produce significantly more dystrophin-positive myofibers than the WT MPCs. These results suggest that genetically modifying donor cells to under-express MSTN could improve the success of cell transplantation.

There have been increasing numbers of biomedical investigators who resort to inhibiting MSTN as a therapeutic strategy for treating muscular dystrophy [11-12, 51]. Among some of the therapies that have been investigated are the use of an anti-MSTN antibody and the use of recombinant MSTN propeptide, both strategies have produced histological and functional improvements in *mdx* mice [11-12]. X. Xiao's group recently reported that the same MPRO cDNA, as used in this study, can be delivered systemically with the AAV-serotype 8 vector to augment the skeletal muscle mass of both normal and *mdx* mice [26]. Moreover, MPRO over-expression resulted in less mononuclear cell infiltration and fibrosis as well as lower creatine kinase levels in the

mdx mice when compared to untreated controls [26]. Interestingly, it has been found that blocking MSTN signal transduction in host *mdx* mice could significantly enhance the muscle regeneration capacity of donor myoblasts [50, 52]. Tremblay *et al* have accomplished this by generating transgenic *mdx* mice with a dominant negative form of MSTN receptor (dnActRIIB), [50] as well as by over-expressing the MSTN inhibitor, follistatin (FLST) [52]. Normal myoblasts transplanted into these transgenic *mdx* mice outperformed cells transplanted in *mdx* mice that were not transgenically modified [50, 52]. Of note is the fact that this data was obtained from experiments using genetically engineered *mdx* mice which makes it difficult to translate these findings for clinical applications. Moreover, dnActRIIB and FLST are not exclusively MSTN-specific inhibitors, as they also block other growth factors such as activin. By contrast to these previous studies, our study examined the impact that would be imparted on the MPCs when injected into the dystrophic muscles of mdx/SCID mice in which MSTN was blocked by AAV-MPRO. Specifically, the AAV-GFP or MPRO constructs were injected into the muscles of *mdx*/SCID mice 4 weeks prior to the transplantation of MPCs in order to investigate whether the AAV-MPRO construct could enhance the muscle regeneration potential of the donor cells by blocking MSTN signaling.

Our results indicate that inactivating MSTN in dystrophic host muscle with MPRO significantly improved the regeneration potential of WT MPC (based on the number of dystrophin positive muscle fibers); when compared to the AAV-GFP transduced controls. We further observed that MPRO ameliorates the dystrophic pathology of *mdx*/SCID mice by reducing collagen deposition, reducing mononuclear cell infiltration, and promoting muscle regeneration. These findings were in sharp contrast to the typical findings of dystrophic muscle, in which muscle fibers are gradually replaced by fibrotic and adipose tissue. It is possible that a favorable microenvironment for donor cells to promote

regeneration is created by inhibiting fibrogenesis. Accordingly, we propose three mechanisms by which cell transplantation can be improved, based on our experimental findings. First, MPRO prevents MSTN from inhibiting donor cell-mediated myofiber regeneration; in doing so, donor cells may be better able to adapt, proliferate, differentiate, and form dystrophin-positive myofibers. Second, MPRO inhibits MSTN, thereby reducing fibrosis and promoting muscle regeneration in dystrophic muscle. Finally, numerous *in vitro* and *in vivo* evidences have demonstrated that typical secondary chronic inflammatory response to progressive muscle necrosis in *mdx* mice are detrimental to healthy muscle cells [53-55] and myofibers [56-57]. The primary onset of muscle necrosis in dystrophin-deficient muscle is primarily attributable to the muscle's increased susceptibility to damage during contraction. Additionally, secondary chronic inflammatory responses further aggravate the symptoms of dystrophinopathies. It has been well documented that an excessive inflammatory response can directly cause severe myofiber damage in both dystrophies and myositis [58-61]. The decrease in degenerative foci, fiber necrosis, and moderated cellular infiltration may suggest that there are fewer cytotoxic cytokines and substances being secreted by inflammatory cells (e.g., neutrophil), such as tumor necrosis factor-α (TNF-α), superoxide, hypochlorite, and nitric oxide [62-64] in the AAV-MPRO-treated dystrophic muscle. Our results indicate that improving the microenvironment of host dystrophic muscle in this fashion probably is a feasible approach for enhancing the efficiency of cell transplantation.

Taken together, the combination of gene and cell therapies may represent a novel, effective approach for treating injured and diseased skeletal muscle. One such specific approach involves the use of the AAV-MPRO to inhibit MSTN in host muscle; an approach that has a potential future use in clinical applications of cellular transplantation therapies.

Figure Legend

Figure 1. AAV-MPRO improves skeletal muscle healing at 4 weeks after injury. (A) Masson trichrome staining shows that fibrotic tissue (blue) extensively exists at the injured site of injured muscles treated with PBS, whereas, fibrotic tissue is mostly limited to the surface of the injured site of AAV-treated injured muscle. (B) Consequently, fibrosis in the AAV-MPRO-treated injured muscle is significantly decreased in comparison to PBS controls. (C) Collagen IV (green) and MPRO (red) double-immunostaining shows strong MPRO signal in the cytoplasm of fibers of AAV-MPRO transduced muscle. PBS injected muscle was used as the negative control. (D) When injured muscles are harvested at 4 weeks after injury, we found that AAV-MPRO tranduced GMs gained significantly more weight over the PBS injected controls. (E) HE staining revealed obvious muscle hypertrophy in the AAV-MPRO-treated muscles. (F) The increase in the diameter of the regenerating myofibers resulting from MPRO is significant (Mean ± SD; ** $P < 0.01$). (G) Frequency histograms show the distribution of regenerating myofibers in AAV-MPRO-treated and control muscles. Scale Bars: 250 μm (A); 100μm (B, C). Original magnifications: x100 (A); x200 (B, C).

Figure 2. AAV-MPRO confers long-term benefits to injured skeletal muscle (1 year after muscle injury). (A) Images show the expression of GFP (green) in the AAV-GFP treated muscle (left panels) at 1 year post injury in contrast to the absence of GFP signal in AAV-MPRO-treated muscle (right panels). Moreover, in the lower panels, collagen IV immunostaining (green) is seen encircling each of the individual muscle fibers. MPRO immunostaining reveals MPRO expression in the cytoplasms of myofibers of AAV-MPRO-treated muscles (right panel), while the AAV-GFP-treated muscle served as a negative control (left panel). (B) Constitutive expression of MPRO in the AAV-MPRO-

treated muscle continues to cause muscle hypertrophy after muscle injury. Larger central nucleated myofibers can be observed in the AAV-MPRO-treated injured muscle as compared to the control. The mean diameter of regenerating myofibers in the former is significantly increased. (C) Distribution of diameters of regenerating myofibers in the AAV-GFP and –MPRO-treated injured muscles. (D) Weight of injured muscles transduced with AAV-MPRO is higher than the control (Mean ± SD; * $P < 0.05$). Scale Bars: 100μm. Original magnifications: x200.

Figure 3. *In vitro* AAV-MPRO transduction. (A) The expression of AAV-MPRO by C2C12 myoblasts was detected by RT-PCR. (B) C2C12 myoblasts were infected with AAV-MPRO. After induction of myogenic fusion in low-serum medium, cells were fixed and stained with MyHC (red); nuclei were stained with DAPI (blue). AAV-MPRO transduction results in the formation of larger MyHC positive multinucleated myotubes and (C) a significant increase in the fusion index (Mean ± SD; * $P < 0.05$). (D) AAV-MPRO stimulated the expression of myogenic regulatory factors, Myf-5 and myogenin, but not MyoD by C2C12 myoblasts. Scale Bars: 250μm. Original magnifications: x100.

~~**Figure 3.** Improved dystrophic pathology in mdx/SCID mice after AAV-MPRO treatment. (A) Small inserts 2x in upper images (HE) show overall morphology of cross-sectional dystrophic muscles. Small arrows in inserts point out poorly stained degenerative foci. More degenerative foci are observed in AAV-GFP-treated dystrophic muscle than in AAV-MPRO-treated dystrophic muscle. When degenerative foci are enlarged, we are able to observe necrotic myofibers (arrowheads) and numerous mononuclear cells (arrows) in AAV-GFP-treated dystrophic muscle, but abundant small newly regenerated~~

~~fibers and less cellular infiltration in AAV-MPRO-treated dystrophic muscle. (B) Masson's trichrome staining reveals that the degenerative foci stain blue, suggesting that there is an accumulation of fibrotic tissue within these foci. The excessive deposition of fibrotic tissue in the foci of AAV-GFP-treated dystrophic muscles can be better appreciated at higher magnification. Measurements of the scar tissue show that AAV-MPRO treatment leads to a significant decrease in fibrosis of dystrophic muscle (Mean ± SD; * $P < 0.01$). (C) The mean diameters of regenerating myofibers in AAV-MPRO-treated muscle is significantly larger than that of AAV-GFP-treated muscle (Mean ± SD; * $P < 0.01$). (D) Moreover, this AAV-MPRO induced muscle hypertrophy resulted in a significant increase in muscle weight (Mean ± SD; * $P < 0.05$). Scale Bars: 500/250 μm (A); 1000/500 μm (B); 100μm (C). Original magnifications: x40/100 (A); x20/40 (B); x200 (C).~~

Figure 4. Muscle progenitor cell characterization and transplantation. (A, E) Desmin immunostaining revealed that the MPC population is highly desmin positive (70.4% positive). (B) In low serum medium, MPCs easily differentiate and fuse into multinucleated myotubes visualized by MyHC (red) and DAPI (blue) staining. (C, E) Flow cytometry indicated 24% of MPCs are Sca-1 positive, 13.5% CD34, and 7% Sca-1/CD34 double positive. (D) *In vivo* characterization shows that MPCs are able to form dystrophin-positive (red) myofibers in dystrophic mice. (F) We injected the same numbers of this MPC population into skeletal muscle of mdx/SCID mice transduced with the AAV-GFP and -MPRO, respectively; the MPCs transplanted into AAV-MPRO transduced muscle regenerated more dystrophin positive myofibers than cells in the GFP control muscle. The increase is significant (Mean ± SEM; * $P < 0.05$). Scale Bars: 250 μm (A, B); 100μm (D, F). Original magnifications: x100 (A, B); x200 (D, F).

Figure 5. Earlier and more neovascularization in the injured MSTN-/- muscle. At 3, 5, 7 days after laceration injury, frozen sections of injured WT and MSTN-/- muscles were stained with HE and double immunostaining: myosin heavy chain (green, MyHC) and CD31 (red), an endothelial marker. Images of HE staining and parallel immunostaining show muscle healing after injury. (A-a, a') At day 3, ruptured myofibers degraded, and numerous mononuclear cells (blue) invaded the injury site. A few residues of necrotic myofibers (black and white arrows) were detected in the injured WT and MSTN-/- muscle. (A-b, b') At day 5, muscle degeneration continued, CD31-positive capillary was absent at the Central Zone of Injury (CZI)(arrowheads). (A-c) At day 7, the CZI of the WT muscle was filled with disorganized connective tissue; capillaries remained absent from the CZI (arrowheads). In contrast, in the MSTN-/- injured muscle (A-c') the CZI possessed some piercing regenerating myofibers (black and white arrows) as well as the in-growth of CD31-positive capillaries. (B) CD31 immunostaining demonstrated that there is a significant increase in CD31 positive capillary-like structures in the injured MSTN-/- muscle compared to the controls (Mean ± SD; ** $P < 0.01$). Scale Bars: 100μm (A, B). Original magnifications: x200 (A, B).

Figure 6. Increased formation of capillary-like structures in AAV-MPRO-treated injured skeletal muscle 4 weeks post-injury. (A) HE staining shows abundant disorganized connective tissue (black arrowheads) in the superficial zone of the injury site of the control muscles, while in the injured AAV-MPRO-treated muscle, regenerating fibers (black arrowheads) have ingrown into the injury site replacing the connective tissue. Inserts are enlarged images of the images in the frames. (B) MyHC (green) and CD31 (red) double immunostaining of parallel slides confirms the absence of myofibers in the injury site (white arrowheads) corresponding to the connective tissue of injured muscle treated with AAV-GFP. Moreover, we are able to observe CD31 positive capillary-like

structures scattered among MyHC-positive regenerating myofibers. (C) CD31 immunostaining (red) indicated a higher density of CD31 positive capillary-like structures in AAV-MPRO-treated injured muscle than that observed in AAV-GFP-treated muscles. The increase in the density of capillary-like structures in the former is statistically significant (Mean ± SD; ** $P < 0.01$). Scale Bars: 250μm. Original magnifications: x100.

Acknowledgements:

The authors would like to thank Dr. Se-Jin Lee for kindly providing breeding pairs of the myostatin knockout mice and Lynne Bauer for maintaining and breeding the *mdx*/SCID and myostatin knockout mice. We would also like to thank James H. Cummins for his editorial assistance in preparing this manuscript.

Funding support

This work was supported by funding from the Henry J. Mankin Endowed Chair for Orthopaedic Research at the University of Pittsburgh, the William F. and Jean W. Donaldson Chair at Children's Hospital of Pittsburgh, the Hirtzel Foundation, the National Institutes of Health (R01 AR47973 awarded to J.H.) and the Department of Defense (W81XWH-06-1-0406 awarded to J.H.).

References

1. McPherron AC, Lawler AM, Lee SJ: Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member, Nature 1997, 387:83-90
2. Acosta J, Carpio Y, Borroto I, Gonzalez O, Estrada MP: Myostatin gene silenced by RNAi show a zebrafish giant phenotype, J Biotechnol 2005, 119:324-331
3. Grobet L, Martin LJ, Poncelet D, Pirottin D, Brouwers B, Riquet J, Schoeberlein A, Dunner S, Menissier F, Massabanda J, Fries R, Hanset R, Georges M: A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle, Nat Genet 1997, 17:71-74
4. Kambadur R, Sharma M, Smith TP, Bass JJ: Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle, Genome Res 1997, 7:910-916
5. McPherron AC, Lee SJ: Double muscling in cattle due to mutations in the myostatin gene, Proc Natl Acad Sci U S A 1997, 94:12457-12461
6. Williams MS: Myostatin mutation associated with gross muscle hypertrophy in a child, N Engl J Med 2004, 351:1030-1031; author reply 1030-1031
7. Pirottin D, Grobet L, Adamantidis A, Farnir F, Herens C, Daa Schroder H, Georges M: Transgenic engineering of male-specific muscular hypertrophy, Proc Natl Acad Sci U S A 2005, 102:6413-6418
8. Lee SJ, McPherron AC: Regulation of myostatin activity and muscle growth, Proc Natl Acad Sci U S A 2001, 98:9306-9311
9. Yang J, Ratovitski T, Brady JP, Solomon MB, Wells KD, Wall RJ: Expression of myostatin pro domain results in muscular transgenic mice, Mol Reprod Dev 2001, 60:351-361
10. Wagner KR, McPherron AC, Winik N, Lee SJ: Loss of myostatin attenuates severity of muscular dystrophy in mdx mice, Ann Neurol 2002, 52:832-836
11. Bogdanovich S, Krag TO, Barton ER, Morris LD, Whittemore LA, Ahima RS, Khurana TS: Functional improvement of dystrophic muscle by myostatin blockade, Nature 2002, 420:418-421
12. Bogdanovich S, Perkins KJ, Krag TO, Whittemore LA, Khurana TS: Myostatin propeptide-mediated amelioration of dystrophic pathophysiology, Faseb J 2005, 19:543-549
13. Zimmers TA, Davies MV, Koniaris LG, Haynes P, Esquela AF, Tomkinson KN, McPherron AC, Wolfman NM, Lee SJ: Induction of cachexia in mice by systemically administered myostatin, Science 2002, 296:1486-1488
14. Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM, Qiu Y: The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum, J Biol Chem 2002, 277:40735-40741
15. Hill JJ, Qiu Y, Hewick RM, Wolfman NM: Regulation of myostatin in vivo by growth and differentiation factor-associated serum protein-1: a novel protein with protease inhibitor and follistatin domains, Mol Endocrinol 2003, 17:1144-1154
16. Lee SJ, Reed LA, Davies MV, Girgenrath S, Goad ME, Tomkinson KN, Wright JF, Barker C, Ehrmantraut G, Holmstrom J, Trowell B, Gertz B, Jiang MS, Sebald SM,

Matzuk M, Li E, Liang LF, Quattlebaum E, Stotish RL, Wolfman NM: Regulation of muscle growth by multiple ligands signaling through activin type II receptors, Proc Natl Acad Sci U S A 2005, 102:18117-18122
17. McCroskery S, Thomas M, Platt L, Hennebry A, Nishimura T, McLeay L, Sharma M, Kambadur R: Improved muscle healing through enhanced regeneration and reduced fibrosis in myostatin-null mice, J Cell Sci 2005, 118:3531-3541
18. Zhu J, Li Y, Shen W, Qiao C, Ambrosio F, Lavasani M, Nozaki M, Branca MF, Huard J: Relationships between transforming growth factor-beta1, myostatin, and decorin: implications for skeletal muscle fibrosis, J Biol Chem 2007, 282:25852-25863
19. Li ZB, Kollias HD, Wagner KR: Myostatin directly regulates skeletal muscle fibrosis, J Biol Chem 2008, 283:19371-19378
20. Rebbapragada A, Benchabane H, Wrana JL, Celeste AJ, Attisano L: Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis, Mol Cell Biol 2003, 23:7230-7242
21. Budasz-Rwiderska M, Jank M, Motyl T: Transforming growth factor-beta1 upregulates myostatin expression in mouse C2C12 myoblasts, J Physiol Pharmacol 2005, 56 Suppl 3:195-214
22. Thomas M, Langley B, Berry C, Sharma M, Kirk S, Bass J, Kambadur R: Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation, J Biol Chem 2000, 275:40235-40243
23. Jiang MS, Liang LF, Wang S, Ratovitski T, Holmstrom J, Barker C, Stotish R: Characterization and identification of the inhibitory domain of GDF-8 propeptide, Biochem Biophys Res Commun 2004, 315:525-531
24. Thies RS, Chen T, Davies MV, Tomkinson KN, Pearson AA, Shakey QA, Wolfman NM: GDF-8 propeptide binds to GDF-8 and antagonizes biological activity by inhibiting GDF-8 receptor binding, Growth Factors 2001, 18:251-259
25. Bartoli M, Poupiot J, Vulin A, Fougerousse F, Arandel L, Daniele N, Roudaut C, Noulet F, Garcia L, Danos O, Richard I: AAV-mediated delivery of a mutated myostatin propeptide ameliorates calpain 3 but not alpha-sarcoglycan deficiency, Gene Ther 2007, 14:733-740
26. Qiao C, Li J, Jiang J, Zhu X, Wang B, Li J, Xiao X: Myostatin propeptide gene delivery by adeno-associated virus serotype 8 vectors enhances muscle growth and ameliorates dystrophic phenotypes in mdx mice, Hum Gene Ther 2008, 19:241-254
27. Xiao X, Li J, Samulski RJ: Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus, J Virol 1998, 72:2224-2232
28. Qu-Petersen Z, Deasy B, Jankowski R, Ikezawa M, Cummins J, Pruchnic R, Mytinger J, Cao B, Gates C, Wernig A, Huard J: Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration, J Cell Biol 2002, 157:851-864
29. Gharaibeh B, Lu A, Tebbets J, Zheng B, Feduska J, Crisan M, Peault B, Cummins J, Huard J: Isolation of a slowly adhering cell fraction containing stem cells from murine skeletal muscle by the preplate technique, Nat Protoc 2008, 3:1501-1509
30. Huard J, Li Y, Fu FH: Muscle injuries and repair: current trends in research, J Bone Joint Surg Am 2002, 84-A:822-832

31. Kaariainen M, Jarvinen T, Jarvinen M, Rantanen J, Kalimo H: Relation between myofibers and connective tissue during muscle injury repair, Scand J Med Sci Sports 2000, 10:332-337
32. Chan YS, Li Y, Foster W, Fu FH, Huard J: The use of suramin, an antifibrotic agent, to improve muscle recovery after strain injury, Am J Sports Med 2005, 33:43-51
33. Chan YS, Li Y, Foster W, Horaguchi T, Somogyi G, Fu FH, Huard J: Antifibrotic effects of suramin in injured skeletal muscle after laceration, J Appl Physiol 2003, 95:771-780
34. Foster W, Li Y, Usas A, Somogyi G, Huard J: Gamma interferon as an antifibrosis agent in skeletal muscle, J Orthop Res 2003, 21:798-804
35. Fukushima K, Badlani N, Usas A, Riano F, Fu F, Huard J: The use of an antifibrosis agent to improve muscle recovery after laceration, Am J Sports Med 2001, 29:394-402
36. Li Y, Negishi S, Sakamoto M, Usas A, Huard J: The use of relaxin improves healing in injured muscle, Ann N Y Acad Sci 2005, 1041:395-397
37. Negishi S, Li Y, Usas A, Fu FH, Huard J: The effect of relaxin treatment on skeletal muscle injuries, Am J Sports Med 2005, 33:1816-1824
38. Sato K, Li Y, Foster W, Fukushima K, Badlani N, Adachi N, Usas A, Fu FH, Huard J: Improvement of muscle healing through enhancement of muscle regeneration and prevention of fibrosis, Muscle Nerve 2003, 28:365-372
39. Bedair HS, Karthikeyan T, Quintero A, Li Y, Huard J: Angiotensin II receptor blockade administered after injury improves muscle regeneration and decreases fibrosis in normal skeletal muscle, Am J Sports Med 2008, 36:1548-1554
40. Eming SA, Brachvogel B, Odorisio T, Koch M: Regulation of angiogenesis: wound healing as a model, Prog Histochem Cytochem 2007, 42:115-170
41. Kalka C, Asahara T, Krone W, Isner JM: [Angiogenesis and vasculogenesis. Therapeutic strategies for stimulation of postnatal neovascularization], Herz 2000, 25:611-622
42. Ahn A, Frishman WH, Gutwein A, Passeri J, Nelson M: Therapeutic angiogenesis: a new treatment approach for ischemic heart disease--Part II, Cardiol Rev 2008, 16:219-229
43. Ahn A, Frishman WH, Gutwein A, Passeri J, Nelson M: Therapeutic angiogenesis: a new treatment approach for ischemic heart disease--part I, Cardiol Rev 2008, 16:163-171
44. Nguyen F, Guigand L, Goubault-Leroux I, Wyers M, Cherel Y: Microvessel density in muscles of dogs with golden retriever muscular dystrophy, Neuromuscul Disord 2005, 15:154-163
45. Okada M, Payne TR, Zheng B, Oshima H, Momoi N, Tobita K, Keller BB, Phillippi JA, Peault B, Huard J: Myogenic endothelial cells purified from human skeletal muscle improve cardiac function after transplantation into infarcted myocardium, J Am Coll Cardiol 2008, 52:1869-1880
46. Payne TR, Oshima H, Okada M, Momoi N, Tobita K, Keller BB, Peng H, Huard J: A relationship between vascular endothelial growth factor, angiogenesis, and cardiac repair after muscle stem cell transplantation into ischemic hearts, J Am Coll Cardiol 2007, 50:1677-1684

47. Musaro A, Rosenthal N: Transgenic mouse models of muscle aging, Exp Gerontol 1999, 34:147-156
48. Chen Y, Luk KD, Cheung KM, Xu R, Lin MC, Lu WW, Leong JC, Kung HF: Gene therapy for new bone formation using adeno-associated viral bone morphogenetic protein-2 vectors, Gene Ther 2003, 10:1345-1353
49. Tal J: Adeno-associated virus-based vectors in gene therapy, J Biomed Sci 2000, 7:279-291
50. Benabdallah BF, Bouchentouf M, Tremblay JP: Improved success of myoblast transplantation in mdx mice by blocking the myostatin signal, Transplantation 2005, 79:1696-1702
51. Bogdanovich S, McNally EM, Khurana TS: Myostatin blockade improves function but not histopathology in a murine model of limb-girdle muscular dystrophy 2C, Muscle Nerve 2008, 37:308-316
52. Benabdallah BF, Bouchentouf M, Rousseau J, Bigey P, Michaud A, Chapdelaine P, Scherman D, Tremblay JP: Inhibiting myostatin with follistatin improves the success of myoblast transplantation in dystrophic mice, Cell Transplant 2008, 17:337-350
53. McLoughlin TJ, Tsivitse SK, Edwards JA, Aiken BA, Pizza FX: Deferoxamine reduces and nitric oxide synthase inhibition increases neutrophil-mediated myotube injury, Cell Tissue Res 2003, 313:313-319
54. Nguyen HX, Tidball JG: Interactions between neutrophils and macrophages promote macrophage killing of rat muscle cells in vitro, J Physiol 2003, 547:125-132
55. Pizza FX, McLoughlin TJ, McGregor SJ, Calomeni EP, Gunning WT: Neutrophils injure cultured skeletal myotubes, Am J Physiol Cell Physiol 2001, 281:C335-341
56. Cheung EV, Tidball JG: Administration of the non-steroidal anti-inflammatory drug ibuprofen increases macrophage concentrations but reduces necrosis during modified muscle use, Inflamm Res 2003, 52:170-176
57. Nguyen HX, Tidball JG: Expression of a muscle-specific, nitric oxide synthase transgene prevents muscle membrane injury and reduces muscle inflammation during modified muscle use in mice, J Physiol 2003, 550:347-356
58. Porter JD, Khanna S, Kaminski HJ, Rao JS, Merriam AP, Richmonds CR, Leahy P, Li J, Guo W, Andrade FH: A chronic inflammatory response dominates the skeletal muscle molecular signature in dystrophin-deficient mdx mice, Hum Mol Genet 2002, 11:263-272
59. Tews DS, Goebel HH: Cell death and oxidative damage in inflammatory myopathies, Clin Immunol Immunopathol 1998, 87:240-247
60. Lundberg IE: The role of cytokines, chemokines, and adhesion molecules in the pathogenesis of idiopathic inflammatory myopathies, Curr Rheumatol Rep 2000, 2:216-224
61. Spencer MJ, Tidball JG: Do immune cells promote the pathology of dystrophin-deficient myopathies?, Neuromuscul Disord 2001, 11:556-564
62. Grounds MD, Torrisi J: Anti-TNFalpha (Remicade) therapy protects dystrophic skeletal muscle from necrosis, Faseb J 2004, 18:676-682
63. Hodgetts S, Radley H, Davies M, Grounds MD: Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFalpha function with Etanercept in mdx mice, Neuromuscul Disord 2006, 16:591-602

64. Pierno S, Nico B, Burdi R, Liantonio A, Didonna MP, Cippone V, Fraysse B, Rolland JF, Mangieri D, Andreetta F, Ferro P, Camerino C, Zallone A, Confalonieri P, De Luca A: Role of tumour necrosis factor alpha, but not of cyclo-oxygenase-2-derived eicosanoids, on functional and morphological indices of dystrophic progression in mdx mice: a pharmacological approach, Neuropathol Appl Neurobiol 2007, 33:344-359

Improving Recovery Following Muscle Injury Using an Angiotensin II Receptor Blocker in both an animal model and in human patients: from the bench to the bedside

[1,2†]Kenji Uehara MD, PhD, [1,2†]Yuri Chun MD, [1,2]Shusuke Ota MD, PhD, [1,2]Tetsuo Kobayashi MD, PhD, [1,2]Sheila J M. Ingham MD, [1,2]Masahiro Nozaki MD, PhD, [4]Kimimasa Tobita MD, [2]James J. Irrgang PT, PhD, [2]Freddie H. Fu MD, [2,3]Vonda Wright MD, [2]Tanya Hagen MD, and [1,2]Johnny Huard PhD

[1]Stem Cell Research Center, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA

[2]Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA

[3]Department of Physical Medicine and Rehabilitation, University of Pittsburgh, Pittsburgh, PA

[4]Department of Developmental Biology, University of Pittsburgh, Pittsburgh, PA

† K.U. and Y. C. contributed equally to this work

Corresponding Author: **Johnny Huard, PhD**

450 Technology Drive, Suite 206
Pittsburgh, PA 15219
Tel: 412-648-2798
FAX: 412-648-4066
e-mail: jhuard@pitt.edu

ABSTRACT

Background: Losartan, an angiotensin II receptor blocker (ARB), has been shown to be a clinically relevant transforming growth factor-β1 (TGF-β1) antagonist in many pathologies. Recently, we have shown that systemic administration of losartan reduced muscle fibrosis and increased the number of regenerating myofibers in an injured skeletal muscle. However, the effective minimum dose of losartan needed to improve muscle healing is still unknown and must be investigated so that this knowledge can eventually be translated into potential clinical use in humans.

Purpose: To investigate the minimum effective dose of losartan that can enhance muscle healing by both stimulating muscle regeneration and preventing fibrosis in an animal model and to evaluate losartan effect on muscle function in two patient who suffered from hamstring muscle injury. We believe that decreased fibrosis secondary to the antagonizing effects of TGF-β1 may expedite recovery.

Study Design: Controlled laboratory Study and Case Series

Methods: *In vitro*: C2C12 cells were cultured with angiotensin II or losartan, and the effect of these substances on myogenic differentiation were investigated.

In vivo: Thirty mice underwent bilateral anterior tibial muscle contusion. Varying concentrations of losartan (3, 10, 30, and 300mg/kg/day) were administrated immediately after injury. The mice were sacrificed 4 weeks after injury. Muscle regeneration and fibrosis were evaluated by histological analysis and functional recovery was measured by physiological testing.

Case studies: Two young male collegiate athletes with acute hamstring muscle injuries were treated with a 30 day course of oral losartan. Muscle strength and flexibility as a measure of function were evaluated over time.

Results: *In vitro*: The myogenic differentiation was attenuated to a minimum of 59.8% compared to control by angiotensin II while losartan stimulated the differentiation to 150.0% compared to control.

In vivo: The effective minimum dose of losartan for the murine model of skeletal muscle injury was 30mg/kg/day administrated immediately after injury in histological analysis. The peak twitch and tetanic forces, at four weeks, of the losartan groups were 21.05±5.26 and 74.16±13.86N/cm^2, respectively, while strength of control group was 12.75±2.51 and 44.78±4.00N/cm^2, respectively.

Case studies: Both athletes showed improved muscle function after losartan treatment was added to standard care. There were no apparent side effects from the medication.

Conclusion: Oral administration of losartan showed significant improvement in muscle healing in a murine model and was well tolerated in two human hamstring injury cases without apparent side effects.

Clinical Relevance: Angiotensin II receptor blockers are clinically available, and may prove to be a safe, well tolerated improvement to current clinical treatment of muscle injury.

Keywords:

Angiotensin II receptor blocker; muscle injury; muscle regeneration; fibrosis

INTRODUCTION

Muscle injuries constitute 10-55% of all injuries in sports medicine [20]. They can be caused by direct injury, including contusions, lacerations and strains, and by indirect injury related to ischemia, neurological dysfunction or medications. Of these, direct force-related injuries account for the majority (60%) [38] of injuries and acute hamstring injuries are very frequent during sports activities [6, 32]. Such injuries are usually managed initially using the RICE (rest, ice, compression, and elevation) protocol, then progressive rehabilitation, eventually including eccentric strengthening. Despite employing what is considered appropriate care, severe muscle injuries typically result in the formation of dense scar tissue (fibrosis) that impairs muscle function, can lead to muscle contracture, chronic pain and diminish the individual's ability to achieve full recovery and return to prior levels of performance.

It is well known that transforming growth factor-β1 (TGF-β1) plays a crucial role in tissue fibrosis [43, 44], particularly in skeletal muscle [1, 5, 26, 45] and warrants attention as a key target for anti-fibrotic applications. With this in mind, we have previously investigated the effects of antagonizing TGF-β1 with decorin[25, 41, 46], relaxin[47], gamma interferon[24], and suramin[12, 52] and have shown their ability to decrease fibrosis and increase regeneration after skeletal muscle injury. Unfortunately, many of the anti-fibrotic agents that have been investigated in the laboratory setting cannot readily be translated to clinical practice due to lack of an oral formulation, Food and Drug Administration (FDA) approval status, and/or a relatively severe side-effect profile.

Losartan is the first orally active, non-peptide angiotensin II (ANGII) type 1 receptor (AT1) blocker (ARB) and is well known as a class of antihypertensive drugs. It

is widely used for treatment of hypertension and congestive heart failure with a low-incidence of adverse effects [18], and is commercially available in an oral formulation. Its TGF-β1 antagonist effect has been shown to be clinically relevant in many other disease states such as renal disease, pulmonary fibrosis, cardiomyopaties, and aortic aneurysm [14, 19, 27, 41, 42]. Additionally, Cohn et al.[8] have shown that losartan can normalize muscle architecture, repair, and function in multiple myopathic states (e.g. Marfan syndrome and Duchenne muscular dystrophy). Moreover, ACE inhibitors have also been shown to improve muscle mass in older patients [12]. Finally, in a murine model of muscle laceration, we demonstrated the up-regulation of AT1 in posttraumatic skeletal muscle tissue [4] as well as the fact that systemic losartan administration reduced muscle fibrosis and increased the number of regenerating myofibers [4]. Functional improvement of the injured muscle has not yet been tested.

In addition to this, the optimal dose of losartan for an anti-fibrotic effect for human skeletal muscle has not yet been determined. The manufacturer's recommended starting dose of losartan for antihypertensive and congestive heart failure therapy is 50mg/day, and clinical doses range from 25mg/day to 100mg/day. However, the effective minimum dose of losartan to improve muscle healing is still unknown. In this study, we sought to investigate the minimum effective dose of losartan necessary to improve skeletal muscle healing after injury in a controlled laboratory setting. We also investigated the effect of the standard losartan dose as adjuvent treatment for acute muscle injury (strain) in two patient-athletes.

MATERIALS AND METHODS

1 – In Vitro Potential of Angiotensin II and Losartan on C2C12 Myoblasts and Fibroblasts

Cell Culture

C2C12 myoblast cells, a well-known myoblast cell line, were purchased from the American Type Culture Collection (Manassas, VA). C2C12 cells were plated at a density of 10,000 cells/well into 12-well plates. The previously published preplate technique was used to isolate fibroblasts from early adhering populations of muscle cells[17, 35]. Isolated fibroblasts were plated at a density of 10,000 cells/well onto 24-well plates. The cells were cultured with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen), and 1% penicillin/streptomycin (P/S) (Invitrogen) until further needed.

Effect of ANGII and Losartan on TGF-β1Expression in Fibroblasts

A TGF-β1 immunoassay (mouse TGF- β1 DuoSet ELISA Development System, R&D systems, Minneapolis, MN) was performed to detect the effect of ANGII and losartan on fibroblasts. After a 24-hour incubation period, the medium was completely removed and replaced with serum-free medium supplemented with a serum replacement (Sigma, St. Louis, MO) containing different concentration of human ANGII (10^{-8}, 10^{-7}, and 10^{-6} mol/L) (Sigma) or losartan (10^{-8} and 10^{-6}mol/L) (Cozaar®; Merck, Whitehouse Station, NJ). After 8 or 24 hour incubations, the medium was collected and the cells were counted by using a hematocytometer. The manufacture's protocol was then used to determine the amount of TGF- β1 in the medium, which was expressed as picogram (pg) of TGF- β1/10,000cells.

Effects of ANGII and Losartan on C2C12 Myoblasts

After a 24-hour incubation period, the medium was removed and replaced by low serum-containing medium (DMEM containing 2% horse serum [HS] [Invitrogen]) containing different concentration of human ANGII (10^{-10}, 10^{-8}, and 10^{-6}mol/L) or losartan (10^{-10}, 10^{-8}, and 10^{-6}mol/L). Medium was replaced with fresh medium (containing the same concentration of ANGII or losartan) every 48 hours. All cells were grown for a total of 5 days. To quantify the differentiation of the C2C12 cells, cells were fixed in cold methanol for 2 minutes and washed in Dulbecco's phosphate-buffered saline (PBS) for 10 minutes at room temperature (RT). Samples were washed twice in PBS, then incubated in blocking buffer (10% HS in PBS) for 30 minutes at RT. Cells were incubated overnight at 4^{o}C with mouse monoclonal anti-skeletal fast myosin heavy chain antibodies (clone MY-32, Sigma) in 2% HS in PBS. After washing with PBS, samples were incubated with the secondary antibody (Alexa Fluor 594 donkey anti-mouse IgG, Invitrogen) in 2% HS for 40 minutes at RT. Cell nuclei were stained with 4', 6-diamidino-2-phenylindole dihydrochroride (Sigma) for 10 minutes at RT. Fusion index (ratio of nuclei in myotubes to all nuclei) was calculated to evaluate cell myogenic differentiation capacity.

2 – In Vivo Evaluation of the Histological and Physiological Effects of Losartan on Muscle Healing After Contusion Injury

Animal Model

The policies and procedures followed for the animal experimentation performed in this study are in accordance with those detailed by the US department of Health and Human Services and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All experimental protocols were approved by the Animal Research and Care Committee of the author's institution. Thirty-three normal mice (C57BL/6J, Jackson Laboratory, Bar Harbor and National Cancer Institute at Frederick, Frederick, MD), aged 8-10 weeks with 21.4 to 25.8g, were used in this experiment.

A muscle contusion injury model was performed, bilaterally, in 30 mice as described previously[25 10 30]. Briefly, the mice were anesthetized with 1.0 to 1.5% isoflurane (Abbott Laboratories, North Chicago, Illinois) in 100% O_2 gas and the animal's hindlimb was positioned by extending the knee and plantar flexing the ankle to 90 degrees. A 16.2g/1.6cm stainless steel ball (Small Parts Inc, Miami Lakes, FL) was dropped from a height of 100cm onto an impactor that reached the mouse's tibialis anterior (TA) muscle. This muscle contusion created is a high-energy blunt injury that results in a large hematoma followed by muscle regeneration and fibrosis[10 25], in a similar fashion as seen in humans[13]. The mice were sacrificed after 4 weeks to evaluate, physiologically and histologically, muscle healing. One-leg of each mouse (6 muscles/group) was assessed physiologically. The contra-lateral hind limb (6 muscles/group) was isolated for histological analysis.

Drug Administration

All injured mice were randomly assigned to 1 of 5 groups (6 mice/group) on the basis of different concentration of oral losartan. The control group was supplied with

normal drinking water while the other 4 treated groups had commercially available losartan (Cozaar®) dissolved in their drinking water at dosage levels of 3, 10 (lower dose groups), 30, or 300mg/kg/day (higher dose groups). These doses were calculated based on the body weight and the average of daily fluid intake which had been monitored once a week before injury. The dose of losartan at 10mg/kg/day is equivalent to the clinical doses for the treatment of high blood pressure (50mg/day) and the dose of 3mg/kg/day is considered lower than minimum clinical dose for human hypertension (25mg/day)[36]. All animals were caged separately and allowed access to the water or losartan solutions *ad libitum* from the time of injury to sacrifice.

Physiological Evaluation of Muscle Strength Recovery after Losartan Therapy

Physiological testing was performed, four weeks after injury (one limb per mouse, n=6 muscles/group), by a modified *in situ* force testing protocol as described by Dellorusso et al.[11] Animals anesthetized with 1.0 to 1.5% isoflurane (Abbott Laboratories) were placed on a heated platform (37^{o}C) and the knee and foot were secured to the platform with cloth tape to better stabilize the hind limb. The TA's distal tendon was exposed, by an incision at the ankle, was sutured securely, as close to the muscle attachment as possible, with a 5-0 silk suture and the tendon was sectioned several milimeters distal to the end of the muscle. The exposed tendon and muscle were kept moist by periodic applications of isotonic saline. After this, the tendon was securely tied to the lever arm of a custom made transducer and data acquisition hardware (NI USB-6215, National Instruments. Austin, TX). The transducer monitored the force

developed by the muscle. All data were displayed and stored on a computer by using a LabView (National Instruments) custom made program.

Anterior crural muscles were stimulated through the peroneal nerve by electrodes inserted via a small incision; the TA muscle was stimulated with 0.2 ms pulses. Stimulation voltage and subsequently muscle length (L_0) were adjusted for maximum isometric twitch force (peak twitch; P_t). While held at L_0, the muscle was stimulated at increasing frequencies, stepwise from 150Hz by 50Hz, until a maximum force (P_0) was monitored. A one- to two-minute rest period was allowed between each tetanic contraction. Then, the entire TA muscle was exposed and the muscle length was measured with a digital caliper. Optimum fiber length (L_f) was calculated by the TA L_f/L_o ratio of 0.6[7]. After the evaluation, the muscle was removed and the mice were euthanized. The tendon and suture were removed and the muscle was weighed. Total muscle cross-sectional area (CSA) of TA muscle was calculated by dividing muscle mass by the product of L_f and 1.06mg/mm^3, the density of mammalian skeletal muscle. Specific P_t and P_0 were calculated by dividinig P_t and P_0 by CSA (N/cm^2). Physiological analysis was also performed in three normal animals as a non-injured group.

Evaluation of Muscle Regeneration after Losartan Therapy

Isolated TA muscles were frozen rapidly in 2-metylbutane pre-cooled with liquid nitrogen. Specimens were kept at -80°C until sectioning. Serial cryosections of 8μm thickness were stained with hematoxylin and eosin staining (H&E) to evaluate the number of regenerating myofibers. The total number of centronucleated myofibers, which were considered as regenerating myofibers[22 37 28], within the injury site was quantified by

using 10 random 200x high-powered fields selected from each sample[16 15 29 30]. Results from the control and each treated groups were compared.

Evaluation of Fibrosis after Losartan Therapy

Masson's Modified IMEB Trichrome stain Kit (IMEB Inc, Chicago, Illinois) was used to measure areas of fibrotic tissue within the injured sites. After staining, the ratio of the fibrotic area to the total cross-sectional area of 10 randomly selected slices was calculated to estimate the fibrosis formation using Northern Eclipse software (Empix Imagine Inc., Cheekatawaga, NY) in accordance with a previously described protocol[16 15 29 30].

Statistical Analysis

All of the results of this study were expressed as the mean±standard deviation. The results of *in vitro* studies were analyzed using Kruskal-Wallis test with Mann-Whitney U-test as post hoc test. To determine minimum effective dose of losartan *in vivo*, we analyzed the results using Williams' multiple comparison. The differences of mean specific twitch and tetanic force between control muscle (injured/non-treated) and normal muscle (non-injured) were analyzed with Kruskal-Wallis test. Statistical difference was defined as $p<0.05$.

3 – Case studies

Two adult males (21 and 22 years old) with acute/recurrent hamstring muscle injury were evaluated and treated with losartan in addition to standard rehabilitation.

Both subjects were healthy college athletes (football and ultimate frisbee) without other injury or co-morbidities.

At the initial evaluation in the clinic, a history and physical examination were performed and each patient-athlete underwent testing that included measurements of isometric hamstring muscle force and flexibility. Isometric force was evaluated with a hand-held dynamometer (Lafayette Instrument Inc); the subject was placed in a prone position and the hand-held dynamometer was placed over the Achilles tendon at the level just proximal to the medial malleolus. Both legs were tested. Three trials were performed with the knee at 30 and 90° of knee flexion. Each trial was held for 3 to 5 seconds as the subject attempted to generate maximal force while the examiner exerted resistance to prevent movement. The averages of the three trials at each angle of knee flexion were calculated. Strength of the injured hamstring muscle was expressed as a percentage of uninjured leg. Flexibility of the hamstrings was measured with a goniometer, with the individual in the supine position, as the amount of available knee extension with the hip flexed to 90°. The patients also had plain radiographs of the pelvis, to rule out fracture of the ischial tuberosity and magnetic resonance imaging (MRI) (1.5T; GE-Sigma, Waukesha, WI, USA) of the involved muscles was performed to better characterize the injury.

After obtaining the patients' consent for treatment, they were started on a 30-day course of losartan (Cozaar®) at the manufacturer's recommended oral daily dose of 50mg. In addition to the medication, the subjects underwent routine standard of care rehabilitation supervised by a physical therapist and/or athletic trainer. The rehabilitation program included the use of superficial heat, flexibility and strengthening exercises for

the hamstring muscles. The strengthening exercises were progressed from isometric to concentric and then eccentric exercises with resistance as tolerated by the athlete. Return to sports activities progressed from jogging to running and sprinting. The patients were evaluated every 7 days by a study physician (YC) that included measurement of blood pressure and hamstring flexibility and strength as described above. At the conclusion of treatment both patients underwent testing on an isokinetic dynamometer (Biodex II; Shirley, New York) at 60 and 180° per second to assess the torque generating capacity of the hamstring and quadriceps muscles.

RESULTS

1 – In Vitro Potential of Angiotensin II and Losartan in C2C12 Myoblasts and Fibroblasts

Effects of ANGII and Losartan on TGF-β1 Expression on Fibloblasts

Fibroblasts, isolated from normal skeletal muscle, up-regulated the expression of TGF-β1, in a dose dependent manner, when cultured in a medium containing 10^{-7} and 10^{-6}mol/L of ANGII at 24 hours after exposure (53.3±0.8 and 89.5±3.3pg/10,000cells, respectively). Both concentrations of losartan (10^{-7} and 10^{-6}mol/L) blocked TGF-β1 expression induced by 10^{-6}mol/L of ANGII (36.9±2.7 and 25.9±2.43pg/10,000cells, respectively) (Figure 1).

Effects of Angiotensin II and Losartan on Myoblasts

The fusion index of C2C12 cells in the groups exposed to 10^{-10}, 10^{-8}, and 10^{-6}mol/L of ANGII were significantly lower (0.23±0.06, 0.27±0.08, and 0.23±0.06,

respectively) than the control group (0.35±0.07). On the other hand, groups treated with losartan (10^{-10}, 10^{-8}, and 10^{-6}mol/L) showed higher fusion indexes (0.17±0.03, 0.16±0.04, and 0.17±0.05, respectively) than the control group (0.12±0.04) (Figure 2).

2 – In vivo Evaluation of the Histological and Physiological Effects of Losartan on Muscle Healing after Contusion Injury

Losartan Improved Muscle Strength Recovery after Contusion Injury.

Results from the physiological evaluations performed at four weeks after injury are shown in Table 1. The control injured group showed significantly less specific peak twitch and tetanic forces (12.75±2.51 and 44.78±4.00N/cm^2, respectively) when compared with the non-injured group (19.24±0.73 and 58.56±11.51N/cm^2, respectively). Specific peak twitch and tetanic forces of higher losartan dose groups (30 and 300mg/kg/day) were greater than control group, while the lower dose groups did not improve muscle strength on specific peak twitch and tetanic forces when compared with control group. The group treated with 30mg/kg/day of losartan showed the best muscular strength recovery on both peak twitch and tetanic forces among the groups. The effective minimum doses for specific P_t and P_0 were 30mg/kg/day group.

Effect of Losartan on Myofiber Regeneration after Contusion Injury

All the centronucleated regenerating myofibers in the injured muscle were counted and compared to control group (Figure 3F). The group treated with 300mg/kg/day of losartan showed the best result among groups (194.52±68.18/hpf) (Figure 3E). The effect of losartan on myofiber regeneration decreased gradually with

reductions in the dose of losartan. The effective minimum dose of losartan on muscle regeneration was 30mg/kg/day (p=0.046). There were no significant differences between lower dose groups (3 and 10mg/kg/day) (108.83±27.25 and 94.47±25.31/hpf, respectively) and control group (91.32±22.95/hpf).

Effect of Losartan on Muscle Fibrosis after Contusion Injury

The area of fibrotic scar tissue was evaluated and compared to the control group (Figure 4F). The mean area of fibrosis of the control group within the zone of injury was 12.80±4.98%. The higher losartan doses groups (30 and 300mg/kg/day) had significantly less fibrosis (9.82±4.93 and 8.48±3.82%, respectively) when compared with control group (p=0.001 and 0.0002, respectively) (Figure 4D, E) while lower losartan doses groups did not show any significant difference.

3 – Case studies

At the time of initial presentation, both patients described a similar hamstring injury approximately 10 weeks prior to the current injury. They had been treated with relative rest and rehabilitation and had returned to full activity without residual symptoms. The remainders of their histories were similar. Exam revealed healthy, active young men who walked without antalgic. Mild ecchymosis was present in the distal posterior thigh, and each had significant tenderness to palpation at the mid substance biceps femoris, without obvious palpable defect. Weakness and pain was reproduced with hamstring manual muscle testing. Neurovascular exam was normal.

X-rays were similar and the MRIs confirmed in both a partial thickness tear of the biceps femoris with surrounding edema. No fracture or significant hematoma was present (Figure 5). Serial isometric strength and flexibility measurements, performed at different time points, are summarized in table 2.

In addition to the isometric tests and isokinetic tests were also performed, the results for the injured side were essentially equal to the uninjured side treated losartan after 9 weeks or 7 weeks. **For patient #1,** at 9 weeks after the injury, the peak hamstring torque of the injured side was 96% at 60°/sec and 107% of the injured side at 180°/sec. In terms of total work performed at 180°/sec there was a 9.8% deficit in total work across the 10 repetitions in comparison to the uninjured side. The patient exhibited more fatigue of the hamstrings on the injured side at 180°/sec. Comparing the work in the 1st 1/3 of the repetitions to the last 1/3 of the repetitions, the decrease in hamstring torque on the injured side was 19% compared to 8.3% on the uninjured side. **For patient #2**, 7 weeks after the injury, peak hamstring torque of the injured side was 92.5% at 60°/sec and 100.4% at 180°/sec compared to the uninjured side. At 180°/sec there was a 6.2% increase in total work across the 10 repetitions that were performed. Contrary to patient #1, fatigue of the injured leg was less than the uninjured leg at 60°/sec (10.3% vs. 17.3%) and at 180°/sec (22.1% vs. 31.2% at 180°/sec).

Both patients reported no side effects while they were taking the losartan and remained normotensive throughout the 30 day course of the medication.

DISCUSSION

This study has shown, *in vitro* and *in vivo* the positive effects of an ARB on muscle healing after injury. We have also demonstrated tolerability and the possibility of improved muscle healing with losartan use in two clinical cases. The *in vitro* section of this study showed that losartan blocks the expression of TGF-β1 and improves muscle cell fusion indexes. We also demonstrated in the *in vivo* studies that systemic administration of losartan immediately after a muscle contusion in a mouse model showed more regenerating myofibers, less fibrotic scar formation, and better recovery in muscular strength when compared with the control group 4 weeks post injury. Moreover, we have described the clinical use of losartan as an adjuvant treatment in healthy athletes with a recurrent grade II (partial tear) hamstring injury. The subjects tolerated the course of losartan well and demonstrated recovery of normal flexibility and strength when compared to the contra-lateral leg.

More than 90% of muscle injuries are caused either by contusion or by excessive strain of the muscle[24]. The morbidity associated with these injuries leads to loss of valuable time in training and competition for an athlete and time lost from work in the occupational patient. Repeat injury is very common. It is known that the structural changes that occur in skeletal muscle after injury contribute to the risk of re-injury [39]. Additionally, it is felt that improved muscle regeneration and reduced fibrosis correlates with enhanced function. Functional recovery after muscle injury is the most important factor for determining the success of therapy and the possibility of translating the technology into clinical application. Our results showed that 30mg/kg/day of losartan was the optimum dose to improve muscle strength in mice when it was administrated immediately after injury. The physiological results corresponded to the effective

minimum dose on histological models as well. Our results suggest that 300mg/kg/day of losartan lead to greater improvements in muscle regeneration and decreased fibrosis when compared to 30mg/kg/day, however, this higher dose of losartan did not seem to be necessary for functional recovery.

As expected to our expectations, the lower dose groups did not show significant improvements in the specific peak twitch and tetanic forces. However, our previous research has shown that muscle healing proceeds through four distinct and sequential phases: necrosis/degeneration, inflammation, regeneration, and finally fibrous tissue formation[20]. The peak of myofiber regeneration and scar tissue formation are approximately 2 and 4 weeks after injury respectively[20, 21], after the peak of capillary generation that occurs at approximately 7 days[31]. Angiogenesis promotes muscle regeneration after the phases of degeneration and inflammation, and is a prerequisite for the subsequent morphological and functional recovery of the injured muscle[23]. Moreover, ANGII plays a critical role to induce angiogenesis in skeletal muscle via AT1[33]. Thus, the angiogenesis might be required for tissue regeneration to activate local rennin angiotensin system (RAS) in the early phase of healing. With these points in mind, in order to provide optimal benefit of the ARB treatment, we postulate ARB therapy should not be immediately initiated after injury but started several days after injury, so that it may reduce ANGII induced apoptosis, differential inhibition of myocytes and tissue fibrosis. Moreover, the ARB therapy must begin prior to deposition of fibrous tissue that occurs approximately 4 weeks after injury. This approach may make it possible to further reduce the effective minimum dose described in this study however additional studies are regular to confirm this. In addition, the contusion injury caused in the animals is massive and

more severe than most of the human injuries found in a sports medicine practice also making it possible to reduce the dose in a human subject.

In both patients we initiated treatment with losartan after the acute period of necrosis/degeneration and inflammation (10 days after injury for the first patient and 5 days after injury for the second patient). Both patients demonstrated normal hamstring flexibility and strength by 9 weeks after injury and were without activity-limiting pain at that time. While meeting this criteria would normally correlate with appropriate time to return to athletics, both athletes suffered injury at the end of their season and thus a return to sport was not a primary end point. To date (> 6 months) neither individual has suffered any further injury to the hamstring muscles, which may represent the most important feature of the study i.e. eliminate potential re-injury.

Extrapolating the time to return to play for hamstring injuries from the previous studies is a difficult and an inexact task as evidenced by the many variables involved and a wide range of time reported in the studies ranging from mean of 14 days to 62 weeks[3 6, 34 40 9 2]. Data on lost time from competition for recurrent hamstring injuries is even more limited and two groups have noted that recurrent injuries prolonged the duration of rehabilitation by 33% (Powell and Zarins, 1989-1998, 1696 injuries in NFL players as reported at 2002 NFLTP meeting) to 79% [6] compared to new injuries. However, these studies reported analysis challenging that compared to longer rehabilitation time for recurrent injuries and new injuries without stratify the severity or location of the injury. For our patients, both would have been able to return to full participation within 9 weeks and in our clinical experience this is a shorter period of time what we have observed in the past for young athletic patients with a similar degree of recurrent injury.

We are encouraged by the results of these interventions; however we recognize the limitations of making treatment decisions based on case reports. We believe that translational clinical research, in the form of a prospective, blinded, placebo controlled randomized clinical trial is necessary to determine the benefits of losartan use in comparison to conventional approaches, to the treatment of hamstring muscle injuries alone. This research should consider the effects of the ARB on recovery of hamstring flexibility and strength, degree of fibrosis, time to return to full participation in sports and the frequency of recurrence as well as any adverse effects encountered by otherwise healthy individuals.

ACKNOWLEDGEMENTS

The authors wish to thank Jessica Tebbets, Joseph Feduska, Michelle Witt MS, Ricardo Ferrari PT MS, Fabrisia Ambrosio PhD, Jinhong Zhu PhD and Burhan Gharaibeh PhD for technical assistance. Funding support was provided by Department of Defense (W81XWH-06-1-0406 awarded to Dr. Johnny Huard), by the William F. and Jean W. Donaldson Chair at Children's Hospital of Pittsburgh, by the Henry J. Mankin Endowed Chair at the University of Pittsburgh, and AFIRM.

REFERENCES

1. Amemiya K, Semino-Mora C, Granger RP, Dalakas MC. Downregulation of TGF-beta1 mRNA and protein in the muscles of patients with inflammatory myopathies after treatment with high-dose intravenous immunoglobulin. *Clin Immunol.* 2000;94(2):99-104.

2. Askling CM, Tengvar M, Saartok T, Thorstensson A. Acute first-time hamstring strains during slow-speed stretching: clinical, magnetic resonance imaging, and recovery characteristics. *Am J Sports Med.* 2007;35(10):1716-1724.

3. Askling CM, Tengvar M, Saartok T, Thorstensson A. Proximal hamstring strains of stretching type in different sports: injury situations, clinical and magnetic resonance imaging characteristics, and return to sport. *Am J Spor ts M ed.* 2008;36(9):1799-1804.

4. Bedair HS, Karthikeyan T, Quintero A, Li Y, Huard J. Angiotensin II receptor blockade administered after injury improves muscle regeneration and decreases fibrosis in normal skeletal muscle. *Am J Sports Med.* 2008;36(8):1548-1554.

5. Bernasconi P, Torchiana E, Confalonieri P, et al. Expression of transforming growth factor-beta 1 in dystrophic patient muscles correlates with fibrosis. Pathogenetic role of a fibrogenic cytokine. *J Clin Invest.* 1995;96(2):1137-1144.

6. Brooks JH, Fuller CW, Kemp SP, Reddin DB. Incidence, risk, and prevention of hamstring muscle injuries in professional rugby union. *Am J Spor ts M ed.* 2006;34(8):1297-1306.

7. Burkholder TJ, Fingado B, Baron S, Lieber RL. Relationship between muscle fiber types and sizes and muscle architectural properties in the mouse hindlimb. *J Morphol.* 1994;221(2):177-190.

8. Cohn RD, van Erp C, Habashi JP, et al. Angiotensin II type 1 receptor blockade attenuates TGF-beta-induced failure of muscle regeneration in multiple myopathic states. *Nat Med.* 2007;13(2):204-210.

9. Connell DA, Schneider-Kolsky ME, Hoving JL, et al. Longitudinal study comparing sonographic and MRI assessments of acute and healing hamstring injuries. *AJR Am J Roentgenol.* 2004;183(4):975-984.

10. Crisco JJ, Jokl P, Heinen GT, Connell MD, Panjabi MM. A muscle contusion injury model. Biomechanics, physiology, and histology. *Am J Spor ts M ed.* 1994;22(5):702-710.

11. Dellorusso C, Crawford RW, Chamberlain JS, Brooks SV. Tibialis anterior muscles in mdx mice are highly susceptible to contraction-induced injury. *J Muscle Res Cell Motil.* 2001;22(5):467-475.

12. Di Bari M, van de Poll-Franse LV, Onder G, et al. Antihypertensive medications and differences in muscle mass in older persons: the Health, Aging and Body Composition Study. *J Am Geriatr Soc.* 2004;52(6):961-966.

13. Diaz JA, Fischer DA, Rettig AC, Davis TJ, Shelbourne KD. Severe quadriceps muscle contusions in athletes. A report of three cases. *Am J Spor ts M ed.* 2003;31(2):289-293.

14. el-Agroudy AE, Hassan NA, Foda MA, et al. Effect of angiotensin II receptor blocker on plasma levels of TGF-beta 1 and interstitial fibrosis in hypertensive kidney transplant patients. *Am J Nephrol.* 2003;23(5):300-306.
15. Foster W, Li Y, Usas A, Somogyi G, Huard J. Gamma interferon as an antifibrosis agent in skeletal muscle. *J Orthop Res.* 2003;21(5):798-804.
16. Fukushima K, Badlani N, Usas A, Riano F, Fu F, Huard J. The use of an antifibrosis agent to improve muscle recovery after laceration. *Am J Sports Med.* 2001;29(4):394-402.
17. Gharaibeh B, Lu A, Tebbets J, et al. Isolation of a slowly adhering cell fraction containing stem cells from murine skeletal muscle by the preplate technique. *Nat Protoc.* 2008;3(9):1501-1509.
18. Goldberg MR, Tanaka W, Barchowsky A, et al. Effects of losartan on blood pressure, plasma renin activity, and angiotensin II in volunteers. *Hypertension.* 1993;21(5):704-713.
19. Habashi JP, Judge DP, Holm TM, et al. Losartan, an AT1 antagonist, prevents aortic aneurysm in a mouse model of Marfan syndrome. *Science.* 2006;312(5770):117-121.
20. Huard J, Li Y, Fu FH. Muscle injuries and repair: current trends in research. *J Bone Joint Surg Am.* 2002;84-A(5):822-832.
21. Huard J, Yokoyama T, Pruchnic R, et al. Muscle-derived cell-mediated ex vivo gene therapy for urological dysfunction. *Gene Ther.* 2002;9(23):1617-1626.
22. Hurme T, Kalimo H. Activation of myogenic precursor cells after muscle injury. *Med Sci Sports Exerc.* 1992;24(2):197-205.
23. Jarvinen M. Healing of a crush injury in rat striated muscle. 3. A micro-angiographical study of the effect of early mobilization and immobilization on capillary ingrowth. *Acta Pathol Microbiol Scand A.* 1976;84(1):85-94.
24. Jarvinen MJ, Lehto MU. The effects of early mobilisation and immobilisation on the healing process following muscle injuries. *Sports Med.* 1993;15(2):78-89.
25. Kasemkijwattana C, Menetrey J, Somogyl G, et al. Development of approaches to improve the healing following muscle contusion. *Cell Transplant.* 1998;7(6):585-598.
26. Li Y, Huard J. Differentiation of muscle-derived cells into myofibroblasts in injured skeletal muscle. *Am J Pathol.* 2002;161(3):895-907.
27. Lim DS, Lutucuta S, Bachireddy P, et al. Angiotensin II blockade reverses myocardial fibrosis in a transgenic mouse model of human hypertrophic cardiomyopathy. *Circulation.* 2001;103(6):789-791.
28. McCormick KM, Schultz E. Role of satellite cells in altering myosin expression during avian skeletal muscle hypertrophy. *Dev Dyn.* 1994;199(1):52-63.
29. Menetrey J, Kasemkijwattana C, Fu FH, Moreland MS, Huard J. Suturing versus immobilization of a muscle laceration. A morphological and functional study in a mouse model. *Am J Sports Med.* 1999;27(2):222-229.
30. Nozaki M, Li Y, Zhu J, et al. Improved muscle healing after contusion injury by the inhibitory effect of suramin on myostatin, a negative regulator of muscle growth. *Am J Sports Med.* 2008;36(12):2354-2362.

31. Ochoa O, Sun D, Reyes-Reyna SM, et al. Delayed angiogenesis and VEGF production in CCR2-/- mice during impaired skeletal muscle regeneration. *Am J Physiol Regul Integr Comp Physiol.* 2007;293(2):R651-661.
32. Orchard J, Seward H. Epidemiology of injuries in the Australian Football League, seasons 1997-2000. *Br J Sports Med.* 2002;36(1):39-44.
33. Petersen MC, Greene AS. Angiotensin II is a critical mediator of prazosin-induced angiogenesis in skeletal muscle. *Microcirculation.* 2007;14(6):583-591.
34. Pomeranz SJ, Heidt RS, Jr. MR imaging in the prognostication of hamstring injury. Work in progress. *Radiology.* 1993;189(3):897-900.
35. Qu-Petersen Z, Deasy B, Jankowski R, et al. Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. *J C ell B iol.* 2002;157(5):851-864.
36. Sasaki M, Uehara S, Ohta H, et al. Losartan ameliorates progression of glomerular structural changes in diabetic KKAy mice. *Life Sc i.* 2004;75(7):869-880.
37. Schultz E, McCormick KM. Skeletal muscle satellite cells. *Rev Physiol B iochem Pharmacol.* 1994;123:213-257.
38. Shanmugam C, Maffulli N. Sports injuries in children. *Br Med Bull.* 2008;86:33-57.
39. Silder A, Heiderscheit BC, Thelen DG, Enright T, Tuite MJ. MR observations of long-term musculotendon remodeling following a hamstring strain injury. *Skeletal Radiol.* 2008;37(12):1101-1109.
40. Slavotinek JP, Verrall GM, Fon GT. Hamstring injury in athletes: using MR imaging measurements to compare extent of muscle injury with amount of time lost from competition. *AJR Am J Roentgenol.* 2002;179(6):1621-1628.
41. Tzanidis A, Lim S, Hannan RD, See F, Ugoni AM, Krum H. Combined angiotensin and endothelin receptor blockade attenuates adverse cardiac remodeling post-myocardial infarction in the rat: possible role of transforming growth factor beta(1). *J Mol Cell Cardiol.* 2001;33(5):969-981.
42. Uhal BD, Kim JK, Li X, Molina-Molina M. Angiotensin-TGF-beta 1 crosstalk in human idiopathic pulmonary fibrosis: autocrine mechanisms in myofibroblasts and macrophages. *Curr Pharm Des.* 2007;13(12):1247-1256.
43. Wynn TA. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. *J Clin Invest.* 2007;117(3):524-529.
44. Wynn TA. Cellular and molecular mechanisms of fibrosis. *J P athol.* 2008;214(2):199-210.
45. Yamazaki M, Minota S, Sakurai H, et al. Expression of transforming growth factor-beta 1 and its relation to endomysial fibrosis in progressive muscular dystrophy. *Am J Pathol.* 1994;144(2):221-226.

Figure legends

Figure 1. TGF-β1 expression in fibroblast at 24 hours after incubation with angiotensin II (10^{-8} mol/L, 10^{-7} mol/L, and 10^{-7} mol/L) and/or losartan. □; 0 mol/L; ■, 10^{-8} mol/L; ■, 10^{-6} mol/L of losartan, respectively. Angiotensin II induced TGF-β1 expression in dose dependent manner (*p < .05). Losartan blocked TGF-β1 expression. *p < .05

Figure 2. Immunocytochemical staining of C2C12 cells for fast myosin heavy chain at 5days after incubation with different concentration of angiotensin II or losartan (A, control; B, 10^{-8} mol/L of angiotensin II; C, 10^{-8} mol/L of losartan). Myotubes are shown in red and nuclei are in blue (original magnification, ×200). D and E, comparison of fusion index of C2C12 differentiation with varied concentrations of angiotensin II or losartan, respectively. *p <.05.

Figure 3. Histologic evaluation of regenerating myofibers at 4weeks after contusion injury by hematoxylin and eosin staining of tibialis anterior muscle treated with different concentration of losartan (A, 0 mg/kg/day; B, 3 mg/kg/day; C, 10 mg/kg/day; D, 30 mg/kg/day; and E, 300 mg/kg/day) initiated immediately after injury. Regenerating myofibers were defined by centronucleated myofibers (original magnification, ×200). The graph (E) depicts quantification in number of regenerating myofibers in losartan treated animals compared with control animals. hpf, high-power field. *p < .05 to control.

Figure 4. Histologic evaluation of the formation of scar tissue at 4weeks after contusion injury by Masson's trichrome staining (collagen: blue, myofibers: red, nuclei: black) of tibialis anterior muscle treated with different concentration of losartan (A, 0 mg/kg/day; B, 3 mg/kg/day; C, 10 mg/kg/day; D, 30 mg/kg/day; and E, 300 mg/kg/day) initiated immediately after injury. The ratio of the fibrotic area to the total cross-sectional area of 10 randomly selected slices was calculated to estimate the fibrosis formation (original magnification, ×100). The graph (E) depicts quantification of fibrotic area in losartan treated animals compared with control animals. hpf, high-power field. *p < .05 to control.

Figure 5. A) Baseline MRI (subject 1) shows a partial tear at the left proximal myotendinous junction (white arrow) of the long head of the biceps femoris; also present is the surrounding edema with high-intensity signal in the hamstring muscle belly.
B) Baseline MRI (subject 2) Grade 2 strain with partial thickness tear of the left biceps femoris at the mid aspect (white arrow), extends approximately 6 cm in the craniocaudal dimension.

Figure 1.

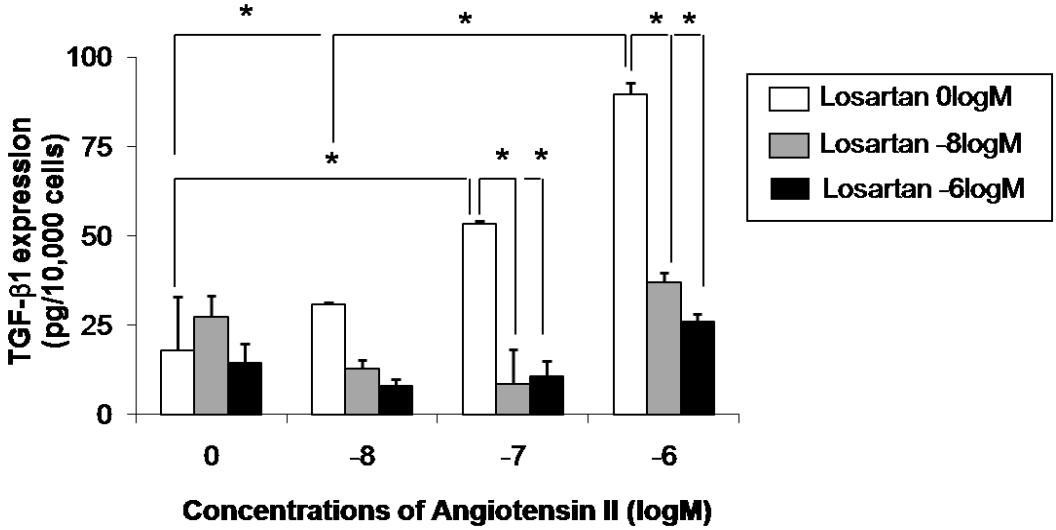

TGF-β1 expression
(pg/10,000 cells)
100
75
50
25
0
0
-8
-7
-6
Concentrations of Angiotensin II (logM)
Losartan 0logM
Losartan -8logM
Losartan -6logM
*
*
* *
*
* *
* *

Figure 2.

Figure 3.

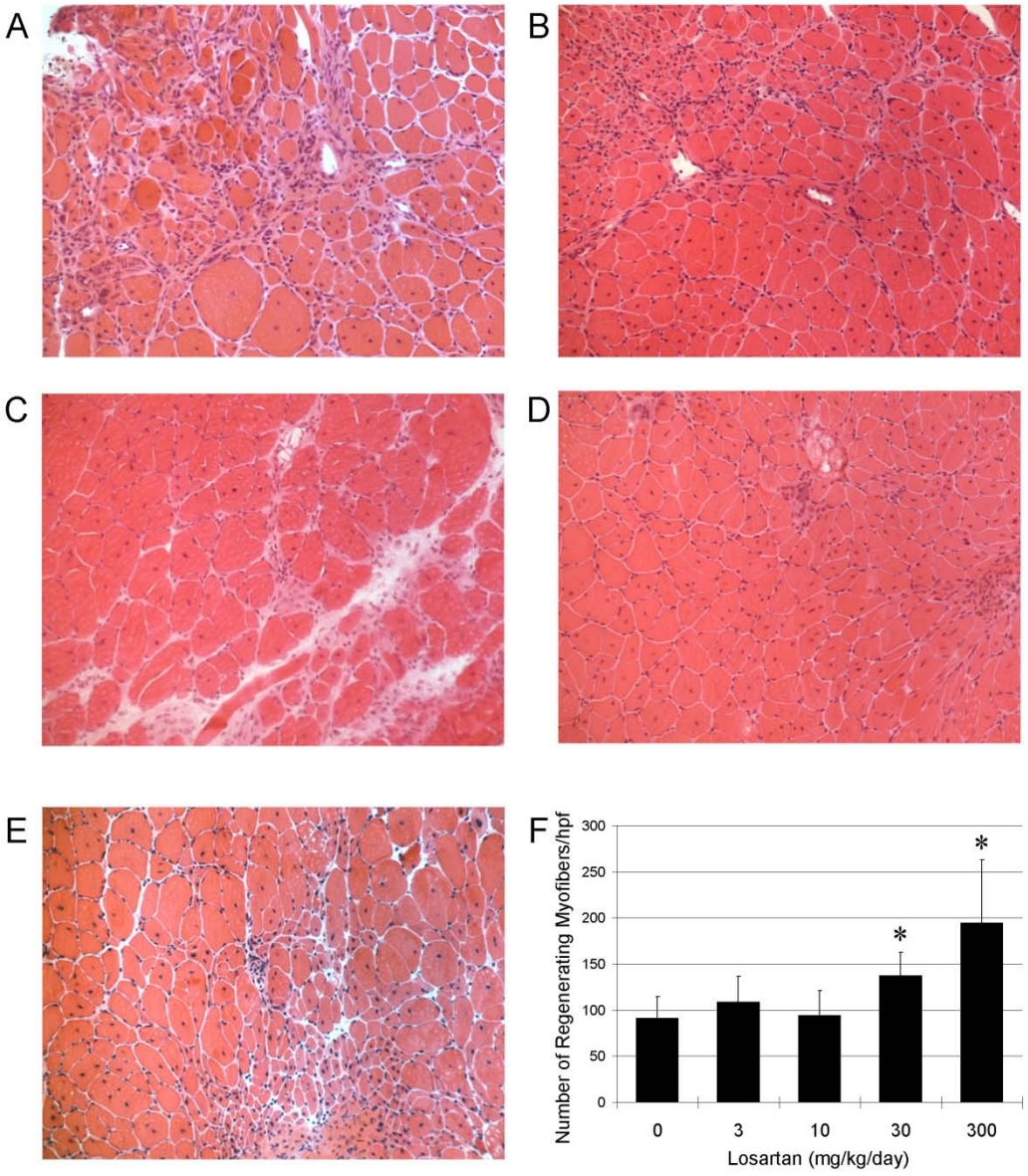

A
B
C
D
E
F
Number of Regenerating Myofibers/hpf
300
250
200
150
100
50
0
*
*
0
3
10
30
300
Losartan (mg/kg/day)

Figure 4.

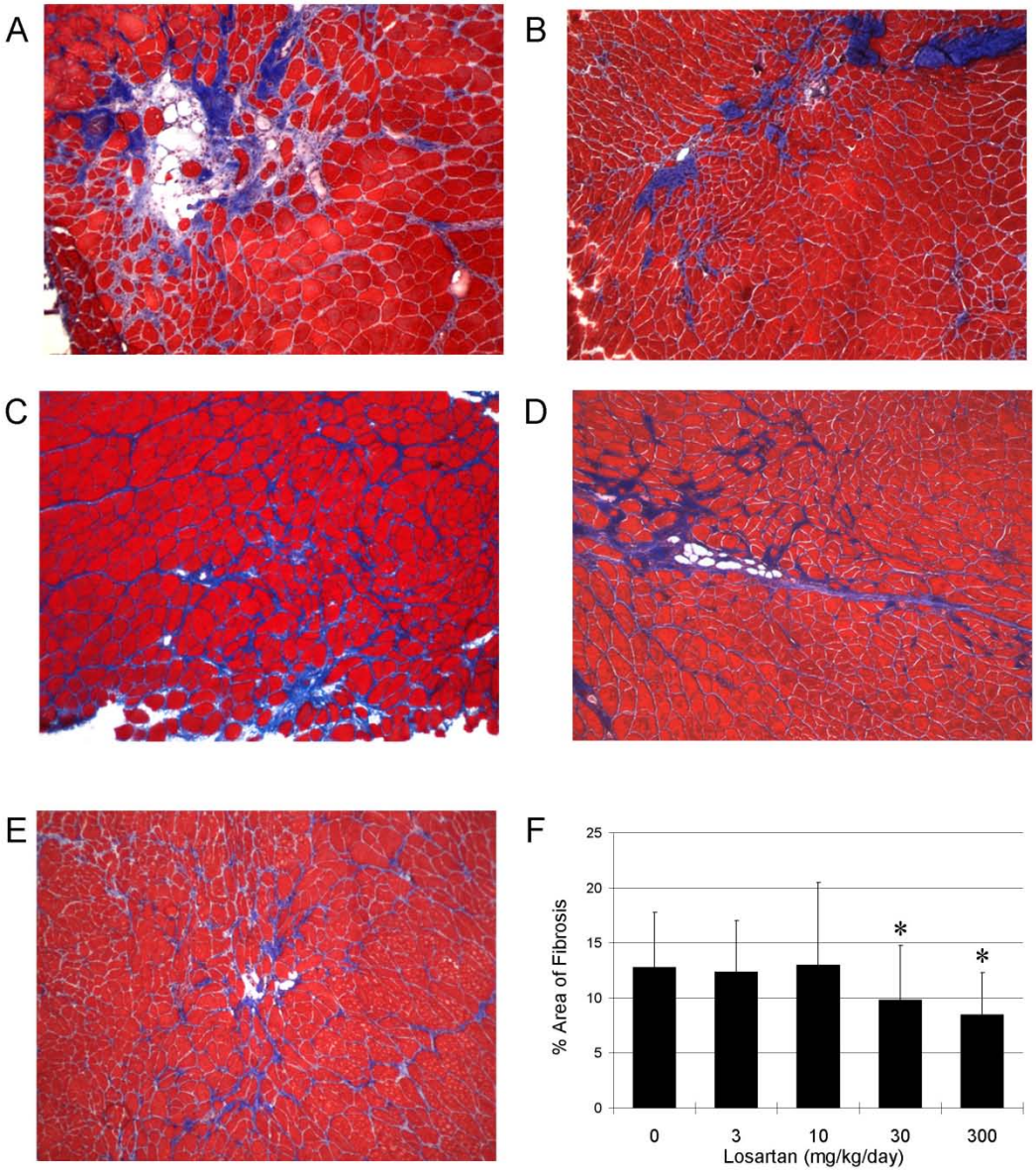

A
B
C
D
E
F
25
20
15
10
5
0
% Area of Fibrosis
*
*
0
3
10
30
300
Losartan (mg/kg/day)

Figure 5

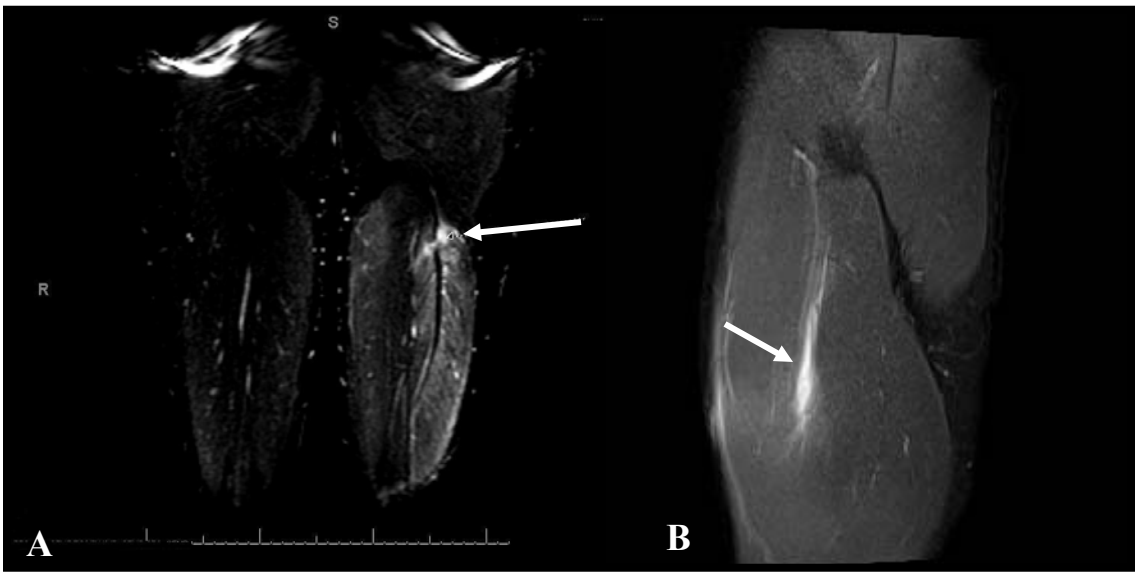


TABLE 1

Results of Specific Peak Twitch and Tetanic Force

Group	Specific Peak Twitch Force (N/cm2)			Specific Peak Tetanic Force (N/cm2)		
normal (noninjured)	19.24	± 0.73		58.56	± 11.51	
control (injured/untreated)	12.75	± 2.51	[b]	44.78	± 4.00	[b]
3 mg/kg/day losartan	13.89	± 2.15		49.46	± 5.32	
10 mg/kg/day losartan	14.45	± 1.42		56.25	± 10.19	
30 mg/kg/day losartan	21.05	± 5.26	[a]	74.16	± 13.86	[a]
300 mg/kg/day losartan	15.80	± 3.77	[a]	67.77	± 20.99	[a]

[a] $p < 0.05$ compared with control group

[b] $p < 0.05$ compared with normal group

TABLE 2

Results of Strength and Flexibility Measurements

	1st evaluation		2nd evaluation		3rd evaluation		4th evaluation		5th evaluation	
	Strength (%)	Pop angle (°)	Strength	Pop angle	Strength	Pop angle	Strength	Pop angle	Strength	Pop angle
Subject 1	47.2 / 54.1	30 (5)	71.0 / 79.6	5 (5)	85.5 / 92.6	5 (5)	91.5 / 83.5	5 (5)	83.3 / 107.1	5 (5)
Subject 2	59.6 / 60.1	13 (10)	101.4 / 85.5	24 (15)	103.8 / 92.5	15 (12)	110.5 / 99.7	26 (18)	132.5 / 110.8	14 (12)

Strength is presented as a percentage of the normal limb. Two values are presented; the first refers to the measurement performed at 30° of knee flexion and the second at 90° of knee flexion. Popliteus angles from both lower extremities are shown as the deficit to full extension; the uninjured side is shown in parentheses. Values shown are an average of three measurements performed.